

# Comparing methods for sensitive determination of Hedgehog active oxysterols

Caroline Vesterdal



Master's degree in chemistry

UNIVERSITETET I OSLO

May 15th, 2015



# Comparing methods for sensitive determination of Hedgehog active oxysterols

Caroline Vesterdal



Master's degree in chemistry

UNIVERSITETET I OSLO

May 15th, 2015

© Caroline Vesterdal

2015

Comparing methods for sensitive determination of Hedgehog active oxysterols

Caroline Vesterdal

<http://www.duo.uio.no/>

Trykk: Reprosentralen, Universitetet i Oslo

# Preface

The work presented in this master thesis was performed at the Department of Chemistry, University of Oslo, from August 2013 to May 2015. My supervisors have been PhD candidate Hanne Røberg-Larsen, Associate Professor Steven R. Wilson, Professor Tyge Greibrokk and Elsa Lundanes.

First of all I would like to thank all my supervisors for an interesting project to work with, for excellent guidance throughout this study and for all the help and feedback while writing this thesis. In addition, I would like to thank Hanne Røberg-Larsen for providing exosome samples.

Thanks to Marita Clausen for supply of equipment and reagents whenever it was needed and thanks to Inge Mikalsen for instrumental troubleshooting and inventions.

Furthermore, I would like to thank past and present members of the Bioanalytical research group for creating a cheerful and amusing social environment. Thanks to Dr. Sofia Lindahl for kind conversations and for challenging work-out sessions. A big thank you also goes to Ole Kristian Brandtzæg for being a good friend throughout the five years of education, for both kind and humorous conversations and for proofreading this thesis. I would also like to thank Gosia for being a splendid office mate.

At the same time, I would like to thank my family and close ones, my fiancé Einar Kvien in particular, for supporting me throughout this educative journey.

Oslo, Norway, May 2015

Caroline Vesterdal

# Abstract

Oxysterols in biological samples of limited size are present in low concentrations thus sensitive methods are advantageous. Derivatization of oxysterols prior to liquid chromatography (LC) mass spectrometry (MS) analysis is a common, however a laborious approach. The possibility to reduce the extent of sample preparation by formation of adducts between oxysterols and mobile phase additives (e.g. ammonium acetate) were investigated. Stable adduct ion signals were not obtained with the use of the MS instruments available. However, loss of water ions ( $[\text{Oxysterol}+\text{H}-\text{H}_2\text{O}]^+$  and  $[\text{Oxysterol}+\text{H}-2\text{H}_2\text{O}]^+$ ,  $m/z$  385.35 and  $m/z$  367.34) were observed when diluting oxysterol standards with ammonium formate (2.5 mM) and formic acid (0.25%) in methanol (MeOH). Under these conditions no easy recognizable fragmentation pattern was observed in tandem MS mode due to clustering of ions in the low mass area when fragmentation energy was applied. Another challenge with the analysis of native oxysterols with a nanoLC system was adsorption to surfaces, especially to fused silica capillaries. Silanization of the fused silica capillaries reduced the issue, but nowhere near satisfactory. It was considered unfeasible to determine native oxysterols with high sensitivity using nanoLC due to the large carry-over issues. Derivatization is therefore recommended when analyzing oxysterols with nanoLC.

An on-line automatic filtration filter back-flush solid phase extraction liquid chromatography tandem mass spectrometry (AFFL-SPE-LC-MS/MS) method for determination of Girard T derivatized 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 22S-hydroxycholesterol was modified for analysis of exosome samples. Best separation of oxysterol isomers was obtained with an ACE 3 C<sub>18</sub> (0.1 mm ID × 150 mm, 3 μm, 100 Å) column with a column temperature of 15°C and by using a mobile phase gradient from 0.1/25/75 (v/v/v %) FA/H<sub>2</sub>O/MeOH to 0.1/10/90 (v/v/v %) FA/H<sub>2</sub>O/MeOH in 25 minutes. The method was used for analyses of exosome samples obtained with the use of different isolation techniques to find a suitable procedure for exosome isolation.

# Abbreviations and definitions

25-OHC	25-hydroxycholesterol
CYP P450	Cytochrome P450
24S-OHC	24S-hydroxycholesterol
27-OHC	27-hydroxycholesterol
CH25H	Cholesterol 25-hydroxylase
Hh	Hedgehog
22S-OHC	22S-hydroxycholesterol
PTC	Patched
SMO	Smoothened
HH	Hedgehog
IHH	Indian Hedgehog
DHH	Desert Hedgehog
SHH	Sonic Hedgehog
mRNA	messenger ribonucleic acid
MVEs	Multivesicular endosomes
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectrometry
ESI	Electrospray ionization

RP	Reversed phase
PFP	Pentafluorophenyl
ID	Inner diameter
APPI	Atmospheric photon ionization
APCI	Atmospheric chemical ionization
Adduct	A product of a direct addition of two or more distinct molecules, resulting in a single reaction product containing all atoms of all components.
MS/MS	Tandem mass spectrometry
SPE	Solid phase extraction
AFFL	Automatic filtration filter back-flush
$m/z$	Mass to charge ratio
AFFL	Automatic filtration filter back-flush
DC	Direct current
RF	Radio frequent
AC	Alternating current
SIM	Single ion monitoring
SRM	Single reaction monitoring
CID	Collision induced dissociation
C-trap	Linear curved trap
HCD	Higher energy collision dissociation
MeOH	Methanol



EtOH	Ethanol
IPA	2-propanol
ACN	Acetonitrile
MP	Mobile phase
FA	Formic acid
AF	Ammonium formate
D	Completeness of the derivatization reaction
M	Matrix effects
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
P/S	Penicillin/Streptomycin Solution
PBS	Phosphate buffered saline
IS	Internal standard
24S-HC-GT	Girard T derivatized 24S-hydroxycholesterol
25-HC-GT	Girard T derivatized 25-hydroxycholesterol
27-HC-GT	Girard T derivatized 27-hydroxycholesterol
22S-HC-GT	Girard T derivatized 22S-hydroxycholesterol
FWHM	Full width half maximum
EIC	Extracted ion chromatogram
PEEK	Polyether ether ketone
T <sub>r</sub>	Retention time

H	Plate height
T <sub>R</sub>	Room temperature
R <sub>s</sub>	Resolution
CE	Exosome samples E72-1mL, E72-3mL and ES (exosomes isolated from medium harvested from cells)
BE	Blank samples B72-1mL, B72-3mL and BS (medium subjected to the same exosome isolation procedure as the medium harvested from cells)
WB	Western blotting
cLOD	Concentration limit of detection
N	Plate number
L	Length
E72h-1mL	Sample obtained by isolation of exosomes with the salting-out procedure. Exosomes were isolated from 1 mL medium (from Sigma) harvested from cells after 72 hours.
E72h-3mL	Sample obtained by isolation of exosomes with the salting-out procedure. Exosomes were isolated from 3 mL medium (from Gibco®) harvested from cells after 72 hours.
ES	Sample obtained by isolation of exosomes with ultracentrifugation. Exosomes were isolated from 50 mL medium (from Gibco®) harvested from cells after 72 hours.
B72h-1mL	Blank sample obtained by isolation of exosomes with the salting-out procedure. Exosomes were isolated from 1 mL medium (from Sigma) harvested after 72 hours.
B72h-3mL	Blank sample obtained by isolation of exosomes with the salting-out procedure. Exosomes were isolated from 3 mL medium (from Gibco®) harvested after 72.

BS	Blank sample obtained by isolation of exosomes with ultracentrifugation. Exosomes were isolated from 50 mL medium (from Gibco®) harvested after 72 hours.
N	Plate number
L	Length

# Table of contents

1	Introduction .....	1
1.1	Oxysterols .....	1
1.2	The Hedgehog pathway .....	2
1.2.1	Exosomes and the Hh pathway .....	4
1.3	Determination of oxysterols .....	5
1.4	Reversed phase liquid chromatography .....	6
1.4.1	Miniaturization of the chromatographic system .....	7
1.4.2	LC-MS analysis of native oxysterols .....	7
1.4.3	Derivatization of oxysterols to enhance ionization efficiency .....	8
1.4.4	Sample clean-up with automatic filtration filter back-flush solid phase extraction combined with LC-MS .....	10
1.5	Electrospray ionization mass spectrometry .....	12
1.5.1	Triple quadrupole mass analyzer .....	13
1.5.2	Orbitrap mass analyzer .....	14
1.6	Aim of study .....	16
2	Experimental .....	17
2.1	Chemicals and standards .....	17
2.1.1	Calibration solutions for quantification of derivatized oxysterols .....	18
2.1.2	Validation solutions .....	18
2.2	Samples .....	19
2.2.1	Isolation of exosomes from cell culture medium with isolation kit .....	19
2.2.2	Isolation of exosomes from cell culture media with ultra-centrifugation ..	20
2.2.3	Derivatization of oxysterols with Girard T reagent .....	21
2.3	Instrumentation .....	22
2.3.1	Direct infusion .....	22
2.3.2	MicoLC system .....	22
2.3.3	AFFL-SPE-nanoLC system I with on-line sample cleanup .....	22
2.3.4	AFFL-SPE-nanoLC system II with on-line sample cleanup .....	23
2.4	Conditions specific for native oxysterol determination .....	25
2.4.1	Silanization of fused silica capillaries .....	25

2.4.2	MS detection.....	25
2.5	Conditions specific for derivatized oxysterol determination .....	26
2.5.1	Off-line sample clean-up of standard solutions for direct infusion .....	26
2.5.2	MS/MS detection .....	26
2.6	Calculations .....	27
2.6.1	Quantification of derivatized oxysterols from calibration curve .....	27
2.6.2	Completeness of the derivatization reaction and matrix effects .....	27
3	Results and discussion .....	28
3.1	Determination of native oxysterols .....	28
3.1.1	Provoking adduct formation to improve ESI-MS detection of native oxysterols .....	28
3.1.2	Ionization of native oxysterols without adduct formation.....	29
3.1.3	Carry-over and adsorption issues of native oxysterols in nanoLC .....	32
3.2	Determination of derivatized oxysterols .....	36
3.2.1	Separation of derivatized oxysterol isomers .....	36
3.2.2	Effect of temperature.....	40
3.2.3	Internal standard.....	41
3.2.4	Examination of carry-over in the AFFL-SPE-nanoLC system .....	43
3.2.5	SPE breakthrough .....	44
3.2.6	Final separation conditions .....	44
3.2.7	Isolation of exosomes .....	45
3.3	Evaluation of method for determination of oxysterols in exosomes .....	45
3.3.1	Selectivity .....	46
3.3.2	Linearity.....	46
3.3.3	Matrix effects .....	47
3.3.4	Completeness of the derivatization reaction and matrix effects .....	48
3.3.5	Cholesterol autoxidation .....	50
3.4	Determination of oxysterols in exosomes .....	51
3.4.1	Evaluation of harvest period, isolation technique and volume culture medium.....	53
4	Conclusion .....	57
5	References .....	58

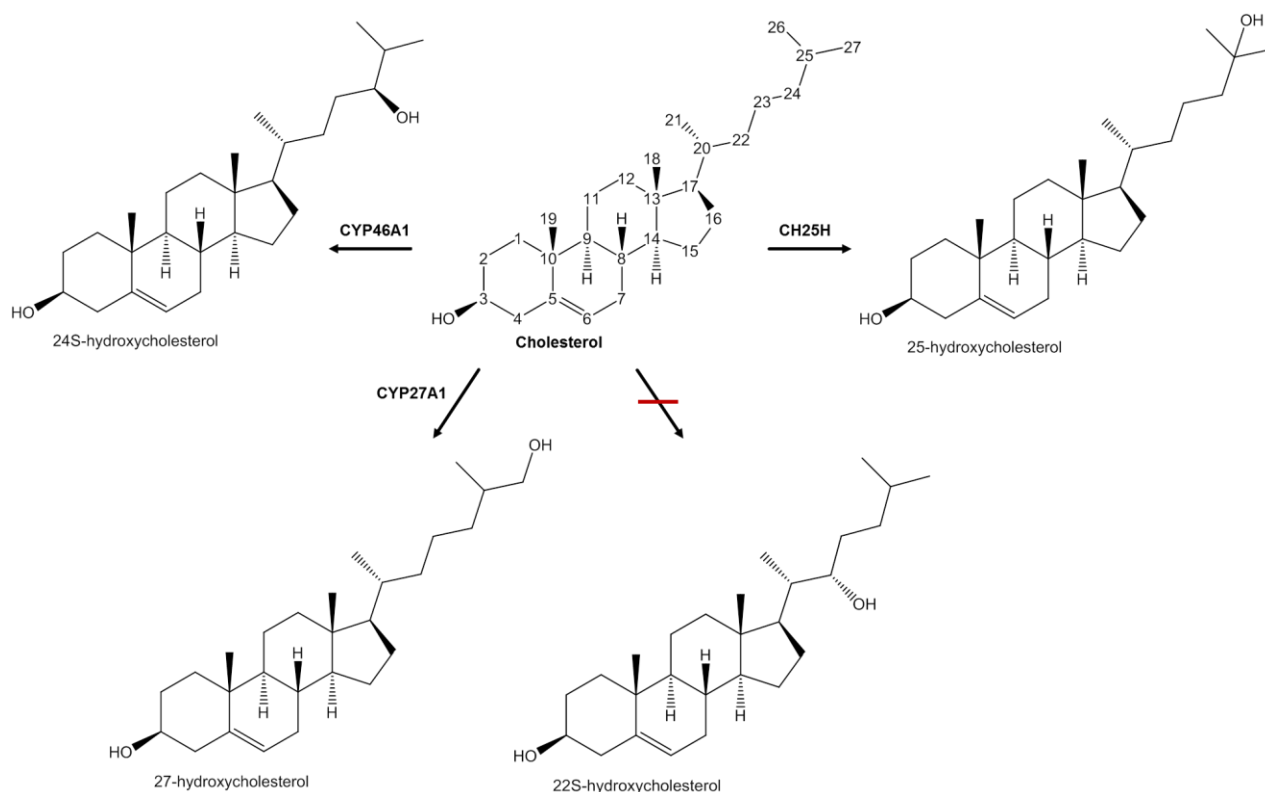
6	Appendix I .....	63
6.1	Signal intensity of acetate adducts of oxysterols .....	63
6.2	Carry-over investigations .....	64
6.2.1	Carry-over in microLC .....	65
6.3	Separation with C <sub>18</sub> -PFP column functionality .....	66
6.4	Increased column length for improved column efficiency .....	67
6.5	Raw data from exosome analysis .....	69
6.6	Investigation of preexisting keto groups .....	70
7	Appendix II .....	72
7.1	Underivatized oxysterols and nanoLC-ESI-MS: A mismatch [82] .....	72
7.2	Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols [65] .....	78

# 1 Introduction

## 1.1 Oxysterols

Oxysterols are biological active molecules that derive from enzymatic or non-enzymatic oxidation (known as autoxidation) of cholesterol [1]. Oxysterols are involved in several biological mechanisms e.g. regulation of cholesterol homeostasis [2], estrogen receptor modulators [3], activating liver X receptors [4], and possibly the Hedgehog signaling pathway [5].

The names of the oxysterols are given by the localization of the hydroxyl group. For instance, if the additional hydroxyl group is in position 25 of the cholesterol molecule the name is 25-hydroxycholesterol (25-OHC). A variety of oxysterols exist, and are mostly formed in the body by metabolizing enzymes belonging to the cytochrome P450 (CYP) family [6]. CYP46A1 converts cholesterol into 24S-hydroxycholesterol (24S-OHC) [7], CYP27A1 produces 27-hydroxycholesterol (27-OHC) [1], while 25-hydroxycholesterol is formed as a by-product of CYP27A1 and by cholesterol 25-hydroxylase (CH25H) [8]. The oxysterols of interest for possible *in vivo* activation of the Hedgehog (Hh) signaling pathway together with their precursor and enzyme are shown in Figure 1. To author's knowledge no known biological enzyme produces 22S-hydroxycholesterol (22S-OHC).



**Figure 1: Structure of the oxidation products of cholesterol formed enzymatically in the body: CYP46A1 forms 24S-hydroxycholesterol, CYP27A1 forms 27-hydroxycholesterol and 25-hydroxycholesterol is formed as a by-product of CYP27A1 and by CH25H. 22S-OHC is a target analyte without a known enzyme (to author's knowledge).**

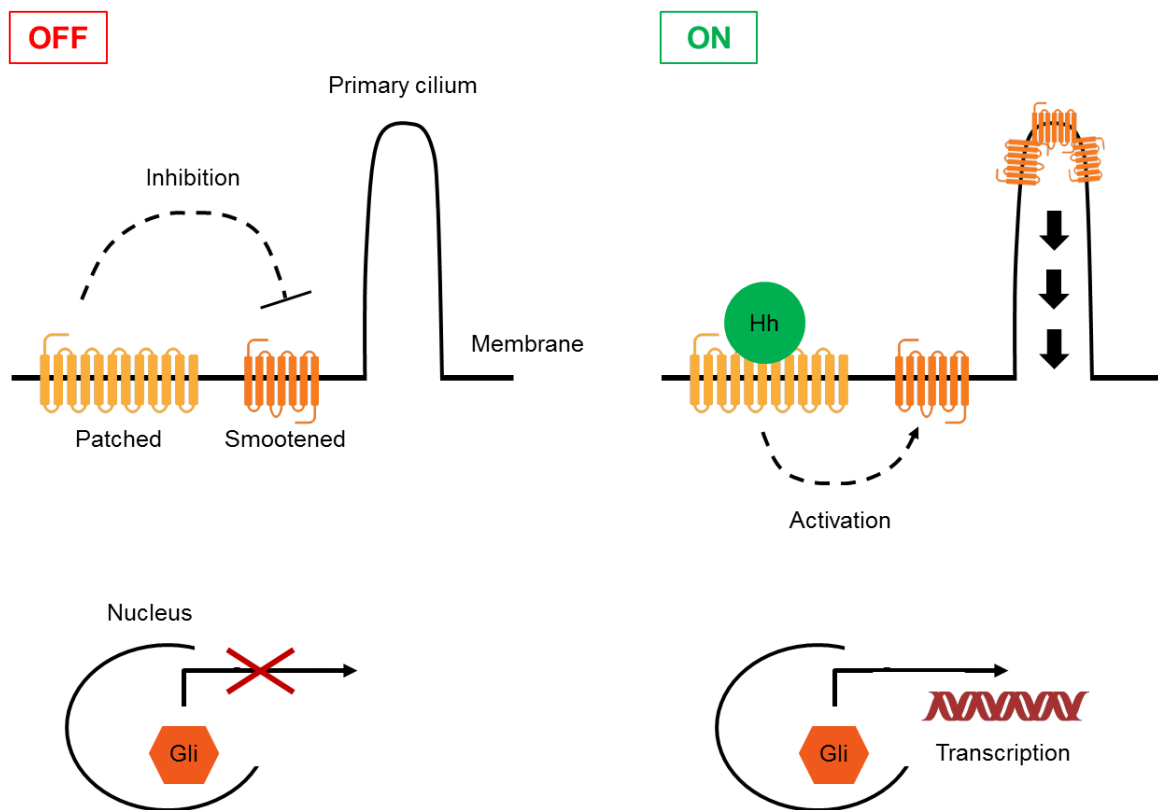
## 1.2 The Hedgehog pathway

It has been reported that oxysterols activates the Hh signaling pathway *in vitro* [9-12], which plays a role in embryonic development [13], stem cells [14], cellular metabolism [15], axon pathfinding and synapse formation [16] and nociception [17]. The pathway is also involved in human disorders and diseases including developmental abnormalities and various forms of cancer [18-24].

Vital players of the Hh pathway were first discovered in the fruit fly *Drosophila Melanogaster* in the late 1970 and early 1980 [25-29]. The key players of the vertebrate pathway are the proteins Patched (PTC) [30], Smoothened (SMO), GLI transcription factors and Hedgehog (HH) [31, 32]. Three different analogs of the HH protein exist, namely Indian Hedgehog (IHH), Desert Hedgehog (DHH) and Sonic Hedgehog (SHH) [5], the latter most



studied. In brief, the pathway (Figure 2) is activated when a HH protein binds to the 12 transmembrane protein, PTC, extracellular on the target cell [10, 11]. After HH binding to PTC, SMO (a 7 transmembrane protein) enters the primary cilium and the GLI transcription factors are activated promoting signaling and initiates gene transcription [10, 11]. In the absence of HH, PTC inhibits SMO by an unknown mechanism, communication is possibly controlled by metabolites [5, 11]. Although natural binding pockets for oxysterols on the extracellular domain of SMO is discovered [9, 11, 12, 33-36] the mechanism for pathway activation with oxysterols *in vivo* is still debated.



**Figure 2: A simplified presentation of the Hh signaling pathway showing the active state (ON) a HH protein binds to PTC, SMO enters the primary cilium and the GLI transcription factors are activated, promoting signaling and initiates gene transcription. And the inactive state (OFF) the HH protein is absent, allowing PTC to inhibit SMO by an unknown mechanism possibly controlled by metabolites.**

### 1.2.1 Exosomes and the Hh pathway

Exosomes are extracellular vesicles containing lipids, messenger ribonucleic acid (mRNA) and proteins [37-39] classified according to size (40-100 nm), density on sucrose gradients (1.13-1.19 g/dL), morphology and molecular constituents [39-41]. The exosomes originate from an early endosome inside the parent cell [40]. The early endosome is divided into multivesicular endosomes (MVEs). These MVEs can either be degraded inside the cell or its content can be released into the extracellular space, as exosomes [40]. These vesicles were formerly considered to only be shed from cells by waste management [42]. Recently, exosomes is reported to be present in a wide range of body fluids [40] and are important in biological mechanisms. The exosomes can potentially be used for prognosis, therapy and as biomarkers for health and disease [37, 41, 43, 44].

In the context of Hh signaling, the HH protein is transported to PTC on a target cell via extracellular vesicles, possibly exosomes [45]. There is also evidence for SHH transport by exosomes specific in size (30-50 nm) [46]. Exosomes are released from normal cells, tumor cells and cancer cells [42, 47], where cancer cells and tumor cells have shown to produce an increased number of exosomes compared with normal cells [47]. Activation of the Hh signaling pathway through exosomes (possibly containing oxysterols) is therefore under investigation.

Because of the recent discovered importance of exosomes, tools to isolate exosomes from bodily fluids are commercially developed and marketed. These tools can potentially improve the isolation efficiency compared with the traditional ultracentrifugation technique [40]. Efficient isolation techniques are important to enable further investigation of the roles of exosomes in biology. In addition, analysis to verify that exosomes has been isolated from other components in bodily fluids is important to enable investigation of the roles of exosomes in the Hh signaling pathway.

## 1.3 Determination of oxysterols

The concentration of oxysterols is low (ng/g- $\mu$ g/g) compared to the abundance of the precursor cholesterol (mg/g) in biological samples [48, 49]; hence sensitive methods are required for their determination. The wide concentration difference between oxysterols and cholesterol in samples puts high demands on the dynamic range of the detector and the chromatographic system. Sufficient sensitivity is a necessity, without overloading the column with cholesterol that might accumulate and make a pseudo stationary phase [50].

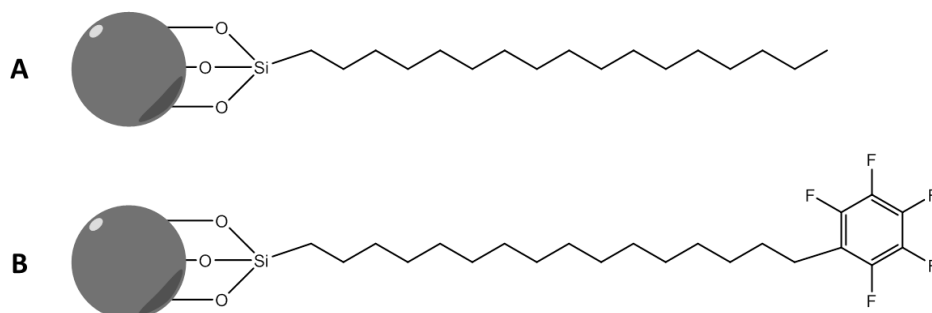
Separation of oxysterol isomers with gas chromatography (GC) or liquid chromatography (LC) is needed prior to mass spectrometry (MS) detection, as the isomers have the same monoisotopic mass (402.35 g/mol). GC-MS is the most used method for quantification of steroids [51-54] and the use of GC requires analytes to be volatile and thermally stable. Oxysterols are not sufficiently volatile for GC analysis and to increase the volatility, derivatization is possible [54]. However, such derivatization procedures often demands laborious procedures with the use of high temperatures. This can promote autoxidation as cholesterol in samples, subjected to light, heat or air, can autoxidize to oxysterols during sample handling or storage [55], giving false positive results. Hence, LC methods can be used for determination of endogenous oxysterols in biological samples [49, 56-58] and have gain popularity the last years. However, neutral oxysterols is less compatible with e.g. electrospray ionization (ESI) since efficient ionization can be difficult. Thus, due to low ionization efficiency in ESI, derivatization is often performed (with e.g. Girard reagents). These reactions are often performed under gentle conditions that do not promote autoxidation.

Possible autoxidation can be monitored with MS by adding heavy cholesterol as an autoxidation standard in both standard solutions and samples [55, 59]. It can be expected that natural and  $^{13}\text{C}$  cholesterol oxidizes if autoxidation occur due to shared characteristics. The heavy oxysterols mass can then be monitored with the use of a MS instrument.

## 1.4 Reversed phase liquid chromatography

Oxysterol isomers can be separated with reversed phase (RP) chromatography, which is the most used separation principle for LC. The stationary phase is hydrophobic and is often silica based with alkyl chains, e.g. C<sub>8</sub> or C<sub>18</sub>, attached. The mobile phase is hydrophilic and mixtures of water or aqueous buffers and water soluble organic solvents are normally used. Elution of analytes from a RP column can be performed with a mobile phase gradient (changing composition of the mobile phase) or isocratically (constant composition of the mobile phase). Gradient elution is commonly employed with complex samples containing analytes with diverse affinity for the stationary phase, while isocratic elution is used for analytes with similar affinity. In gradient elution, the amount of organic solvent (with higher elution strength compared with water or an aqueous buffer) is gradually increased. Thereby, more hydrophobic compounds elutes from the column due to reduced interaction between analyte and stationary phase.

Other phases with different selectivity, e.g. C<sub>18</sub>-pentafluorophenyl (PFP) can be attached to the silica based surface. The PFP functionality may be suited when e.g. separating regioisomers or analytes with differing shape constraints [60], such as oxysterols. The separation mechanisms can be based on hydrophobic,  $\pi$ - $\pi$  interactions, dipole-dipole, hydrogen bonding and shape selectivity depending on the physical and chemical properties of the analytes, its structure and chromatographic conditions [60]. The C<sub>18</sub> and C<sub>18</sub>-PFP stationary phases are presented in Figure 3.



**Figure 3: Column functionality of (A) a C<sub>18</sub> stationary phase and (B) a C<sub>18</sub>-PFP stationary phase.**

### 1.4.1 Miniaturization of the chromatographic system

One possible way to achieve high sensitivity in LC is to reduce the inner diameter (ID) of the column. With narrower columns, a low flow rate is used giving less chromatographic dilution, hence more concentrated eluting peaks in combination with concentration sensitive detectors (e.g. ESI-MS), thus the sensitivity will increase.

A chromatographic system with narrow columns hence low flow rate is also more compatible with ESI, making flow splitting between column and the MS inlet unnecessary. Also, less mobile phase solvent is consumed, which is favorable for the environment and economy. However, there are some limitations with miniaturized chromatographic systems: the use of narrow capillaries (with low volume) is needed to connect instrumental parts. These systems are less robust and clogging may be a frequent issue. Furthermore, proper couplings are essential to reduce dead volumes to avoid band broadening of eluting peaks.

### 1.4.2 LC-MS analysis of native oxysterols

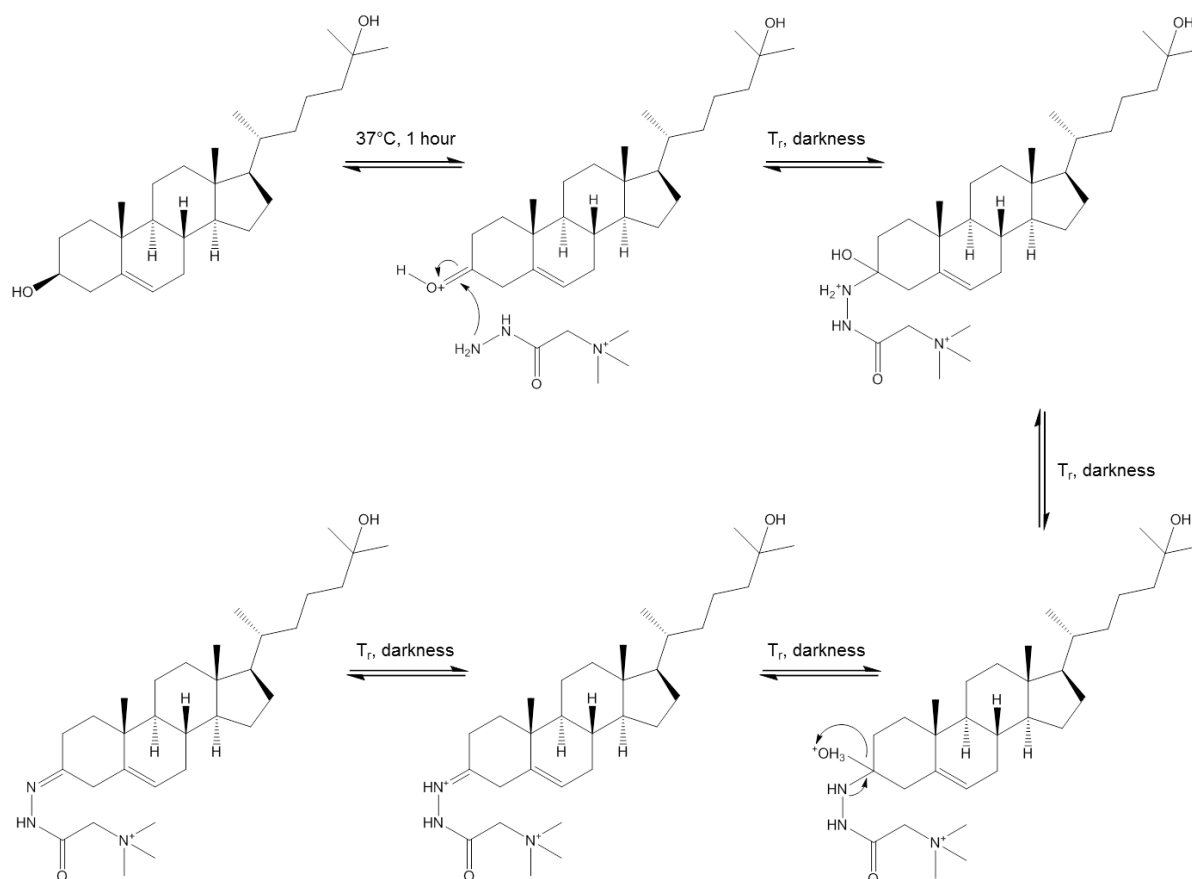
Since sterols are neutral and regarded as non-ionizable compounds, alternative ionization sources such as atmospheric photon ionization (APPI) or atmospheric pressure chemical ionization (APCI) can be better suited. By using these ion sources in combination with MS, oxysterols have been determined through loss of water ions as  $[\text{oxysterol} + \text{H} - \text{H}_2\text{O}]^+$  or  $[\text{oxysterol} + \text{H} - 2\text{H}_2\text{O}]^+$  [58, 61, 62]. Nevertheless, these mass sensitive ionization sources are more compatible with higher flow rate, hence less suitable for nanoLC. Concentration sensitive detectors as ESI-MS can therefore be more appropriate with low flows in combination with narrow columns for sensitive determination of low abundant analytes (as oxysterols).

The solvents used in the chromatographic system needs to dissolve the hydrophobic native oxysterols in addition to promote ionization in ESI. To attain charge, additives as ammonium acetate can be added in the mobile phase to create adducts prior to ESI-MS. With this approach, adducts between oxysterol and ammonia ( $[\text{oxysterol}+\text{NH}_4]^+$ ) have been used to determine a wide range of sterols [63, 64]. However, ionization and formation of adducts between sterol and mobile phase ions is reported to be dependent on both ionization source and mass spectrometer [64].

### 1.4.3 Derivatization of oxysterols to enhance ionization efficiency

Oxysterols can be detected efficiently with ESI-MS after charge tagging with a derivatization agent e.g. Girard reagents [55, 59, 65-67], picolinyl ester [68, 69] or N,N dimethylglycine esters [70]. Derivatization reactions often include several hands-on steps which increases the risk of analyte loss. This can be minimized by performing the procedure in one tube [65]. Derivatization with Girard T (or Girard P) reagent gains a high yield of oxysterols with a positive charge (Figure 4). Girard reagents are specific for oxo groups and by using the enzyme cholesterol oxidase, the hydroxyl group (in position 3 of the oxysterol molecule) is oxidized to a keto group. Secondly, the hydroxyl group is replaced with a hydrazine group. A potential pitfall with this procedure is that the Girard T reagent can react with analyte analogues with naturally occurring keto groups. Keto groups formed during sample preparation and analyte analogs with preexisting keto groups will therefore be detectable [65].

Introduction of the hydrazine group makes the oxysterol species charged and more polar. This will enhance the solubility of oxysterols in RP mobile phases in addition to enhance the ionization efficiency in ESI [65, 67, 71]. Unfortunately, the derivatization reaction generates syn and anti-formations within the molecule (with regard to the hydrazine group), which may complicate the chromatography as peak broadening/splitting is observed for some chromatographic conditions [59, 65].



**Figure 4: Derivatization reaction of oxysterol with Girard T reagent, here shown with 25-OHC. Initially, the hydroxyl group (in position 3) is transformed to a keto group by enzymatic oxidation with cholesterol oxidase at 37°C for 1 hour. Subsequently, the oxidized oxysterol react with the Girard T reagent in room temperature (T<sub>R</sub>) in the dark overnight resulting in Girard T derivatized oxysterol.**

With a mass analyzer operated in tandem MS (MS/MS) mode derivatized oxysterols can be fragmented and thereby be identified more selectively [56]. A possible fragmentation pattern of Girard T derivatives of oxysterols is shown in Figure 5.

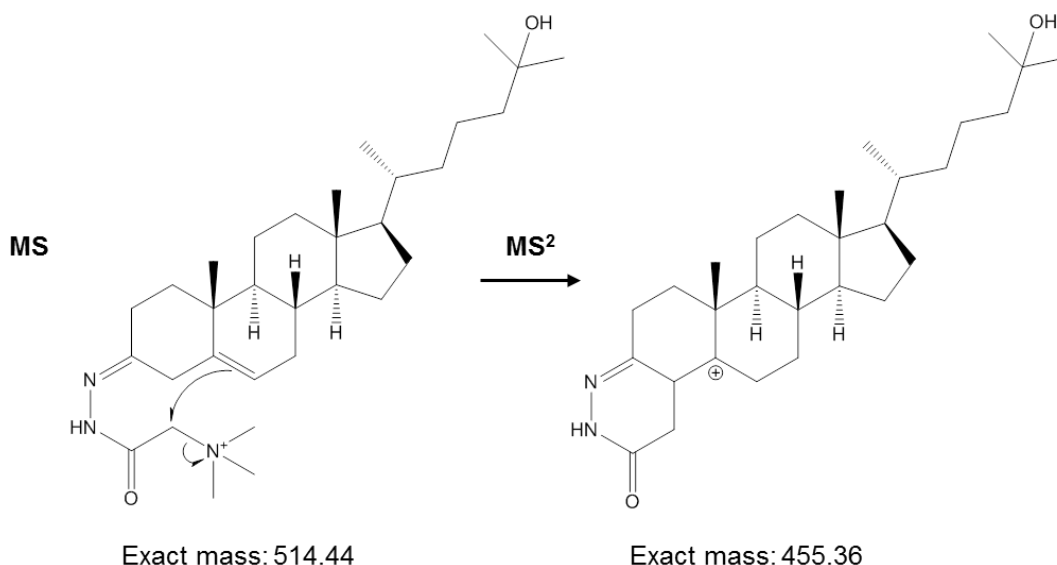


Figure 5: Possible fragmentation pattern of Girard T derivatives of oxysterols in MS/MS [72].

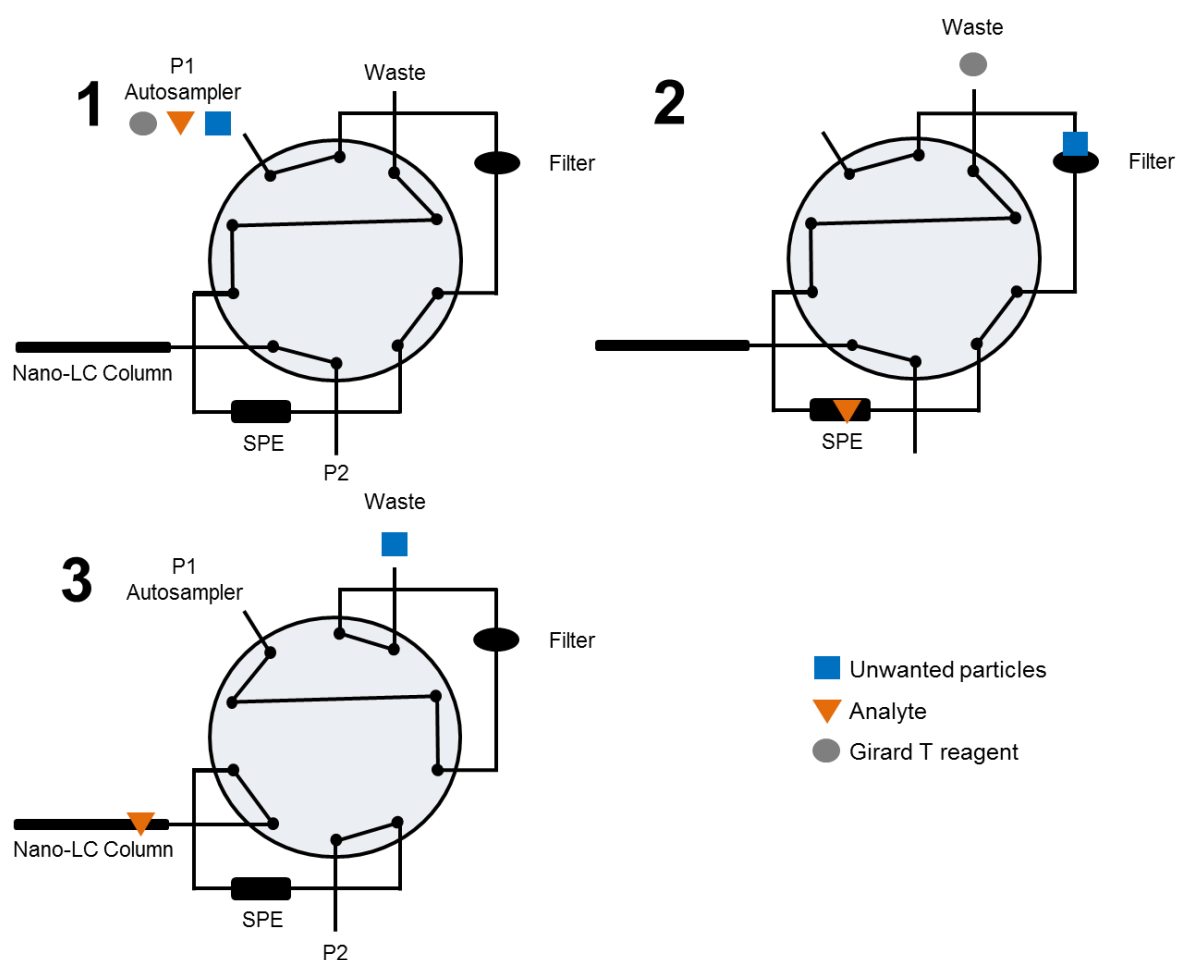
#### 1.4.4 Sample clean-up with automatic filtration filter back-flush solid phase extraction combined with LC-MS

Clogging of the narrow column in miniaturized systems by e.g. particles from the sample and overloading of the column with sample matrix can be an issue. Derivatization methods often include addition of excess reagents and/or solvents. To obtain a clean sample, free from particles and superfluous additives, sample clean-up can be performed on solid phase extraction (SPE) columns either manual (off-line) or by the LC-system (on-line). The aim is to pre-concentrate the analytes by removing matrix in addition to remove particles to avoid clogging. Sample clean-up performed off-line requires several manual steps which can affect the precision and reproducibility of the results negatively [59]. An on-line approach reduces manual efforts, can be less time-consuming and the risk of sample loss and contamination decreases. An on-line method such as SPE-LC is effective, but can also be associated with pressure built up and clogging issues. Pressure build-up can be caused by accumulated particles on the SPE column, hence additional manual steps as off-line filtration and/or off-line SPE procedures are often performed [73]. This can be time-consuming and again increases the risk of sample loss and contamination of the sample.

Alternatively, filtration can be performed on-line in combination with SPE with an automated filtration filter back-flush (AFFL)-SPE system [73] shown in Figure 6. The sample containing



unwanted particles, analytes and other substances pass through a stainless steel filter prior to the SPE column. The filter traps particles whereas analytes are trapped on the SPE column. Non-retaining substances pass through the SPE and are washed out to waste. Subsequently, the valve is switched and analytes elute onto the analytical column for separation, while a second pump washes off particles from the stainless steel filter. This ensures that the filter, SPE and LC columns will not clog and a reasonable pressure in the system will be maintained, hence the robustness of the method improves [59, 73].

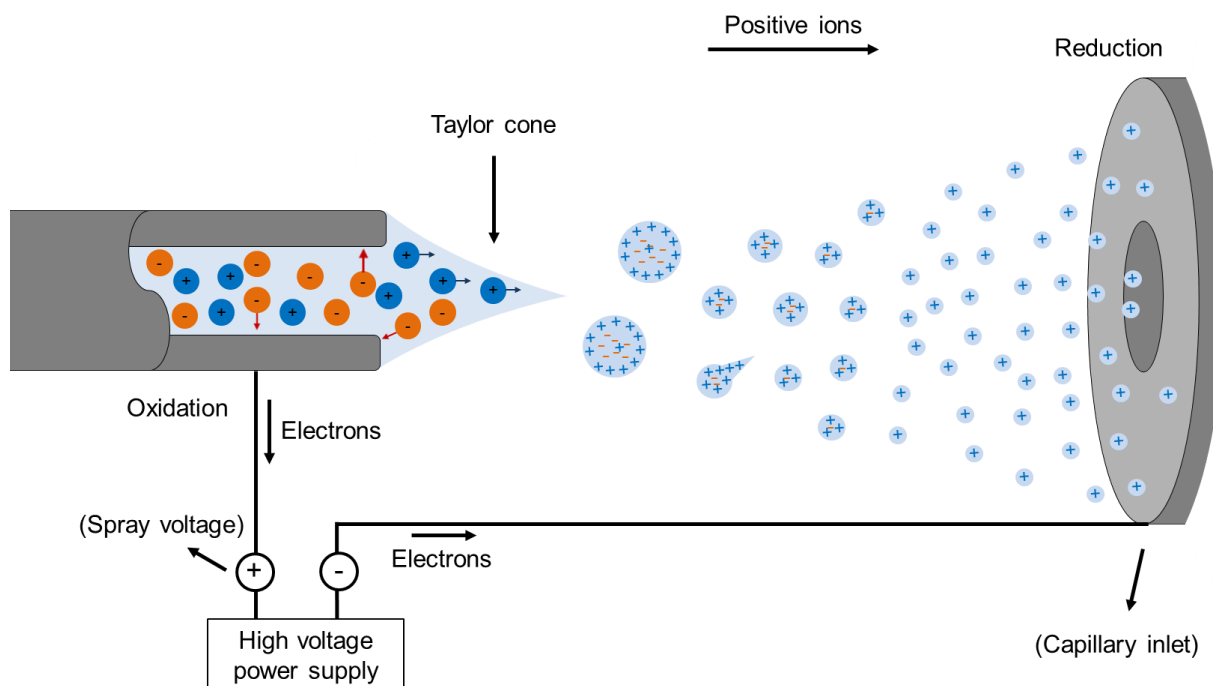


**Figure 6: Schematic of an AFFL-SPE-LC system performed with a 10-port two-position switching valve. (1) Sample containing excess of Girard T reagent is injected by an autosampler. (2) A filter traps unwanted particles while analytes are trapped on the SPE and reagent is flushed to waste. (3) After switching the valve, the analytes are transferred to the analytical column for separation and simultaneously the filter is back-flushed. Adapted from [65].**

## 1.5 Electrospray ionization mass spectrometry

ESI is mostly used for ionizable (acidic or basic) compounds in LC-MS, where ions are generated in the mobile phase solution by adjusting the pH. It is highly compatible with nanoLC, as a small volume from the spray tip allows more efficient generation of gas phase ions [74]. Greater sensitivity is achieved, which is advantageous when studying low abundant analytes in limited biological samples. In addition, ESI is a soft ionization technique giving little fragmentation [75], because energy is transferred gradually to the analyte by thermal energy at low temperatures. Hence, the biomolecule ion is preserved from solution to gas phase.

In ESI, the ions in solution are transferred to gas-phase ions in the atmospheric region of the instrument as illustrated in Figure 7. The ESI capillary tip is subjected to high voltage and opposite charges are attracted to the capillary while molecules with the same charge pass further due to repulsions and attractions towards an opposite charged electrode [74, 75]. At a specific voltage a Taylor cone forms and charged droplets (close to their Rayleigh limit) are generated [74]. The droplets start to evaporate generating smaller droplets with accumulated charge. Cycles of solvent evaporation and droplet fission transfer liquid ions to gas phase [74, 75].

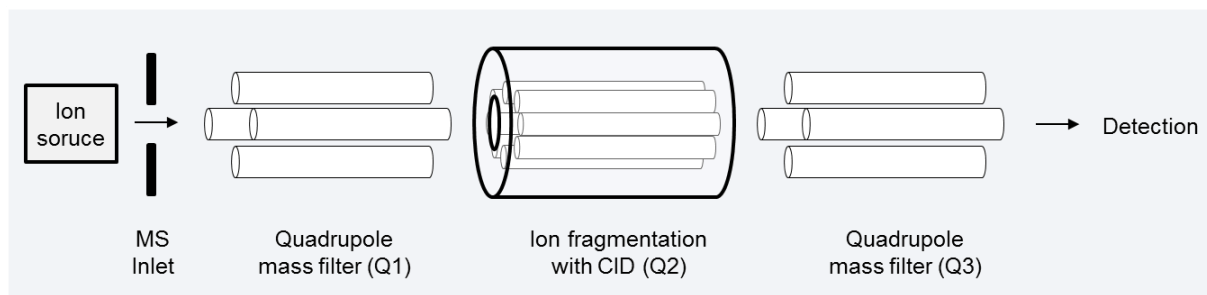


**Figure 7: Electrospray process showing positive ion formation in the atmospheric region of the instrument. A Taylor cone is formed from the spray tip with subsequent formation of charged droplets attracted towards and opposite charged electrode. Cycles of evaporation and droplet fission transfer liquid ions to gas phase [76].**

### 1.5.1 Triple quadrupole mass analyzer

The mass analyzer separates the gas phase ions with high resolution and high sensitivity. With high resolution, smaller mass to charge ratio ( $m/z$ ) differences can be separated. The most used mass analyzer for quantitative purposes is the triple quadrupole, shown in Figure 8. It is built up of two mass filters (Q1 and Q3) each consisting of a pair of rods with opposite potential. The rods are connected electrically, one pair attached to a variable positive direct current (DC) source and the other to the negative DC source. To each pair of rods a variable radio-frequent (RF) alternating current (AC) potential are applied. Q1 and Q3 are controlled by varying the AC and DC potential. Depending on the AC/DC potential ions with a certain  $m/z$  ratio are allowed to go through the triple quadrupole and be detected. Different scan types e.g. single ion monitoring (SIM) and single reaction monitoring (SRM) can be performed. The latter is used to obtain enhanced selectivity and sensitivity. With SRM, Q1 allows a certain  $m/z$  (precursor ion) to enter the collision cell (Q2), consisting of a multipole, where the

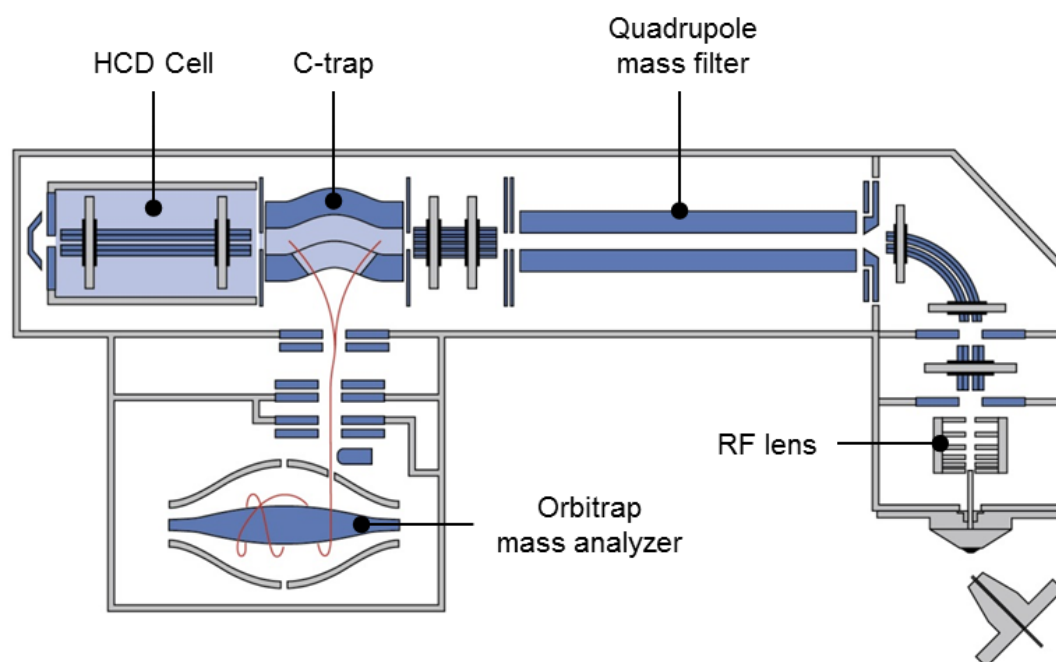
ion is fragmented by collision induced dissociation (CID). Molecular ions are accelerated by electrical potential to high kinetic energy and collide with neutral molecules e.g. He, N or Ar. The precursor ion is fragmented and a product ion forms. By selecting the product  $m/z$ , the product ion is allowed to pass through Q3 and be detected. In this way, only specific precursor ions with specific product ions are detected.



**Figure 8: Schematic of a triple quadrupole mass analyzer consisting of two mass filters (Q1 and Q3) in addition to a collision cell where ions can be fragmented by collision with neutral molecules.**

### 1.5.2 Orbitrap mass analyzer

An Orbitrap mass analyzer (Figure 9) is attributed high mass accuracy and high resolution and are mostly used in proteomics or discovery analysis (unknown analytes). Ions are trapped in a curved linear trap (C-trap) which ejects ion packets into an Orbitrap. Ion packets are ejected by reducing RF voltage and applying DC potential across the C-trap. Ions enter the central electrode where electrostatic attraction is compensated by centrifugal forces [77]. The electrostatic field forces ions to oscillate in discrete orbits according to their  $m/z$  before detection [77]. To perform MS/MS the Orbitrap is connected with a quadrupole mass filter and a collision cell, which is allied with the C-trap. Ions can be fragmented by higher-energy collisional dissociation (HCD) which takes place external to the trap. Ions return to the C-trap before re-injected to central electrode for  $m/z$  separation. Different scan types can be performed, in SIM scans; selected  $m/z$  values are detected. SIM scan can be performed with MS or MS/MS.



**Figure 9: Schematic of a Q-Exactive™ hybrid quadrupole Orbitrap mass analyzer. Ion packets from the C-trap are ejected to the Orbitrap mass analyzer where the voltage is ramped and ions according to their  $m/z$  are separated in discrete oscillating paths. Detection occurs and the signal is amplified. Adapted from (Thermo Scientific) [78].**

## 1.6 Aim of study

The aim of this study was to develop a simple and sensitive method using nanoLC for determination of native oxysterols by exploring the possibility to form adducts between oxysterol and mobile phase additives. The purpose of employing adducts for determination of oxysterols was to reduce the time and complexity of the sample preparation steps employed in our established derivatization method [65].

The developed method was to be compared with the established method using Girard T derivatives of oxysterols for determination. The most sensitive method should be employed for determination of possible oxysterols in exosomes from cancer cells, for further exploration and understanding of the Hh pathway.

## 2 Experimental

### 2.1 Chemicals and standards

Mixtures of HPLC grade methanol (MeOH, VWR, Radnor, PE, US) or LC-MS Chromasolv® grade MeOH (Fluka, Sigma Aldrich, St. Louis, MO, US), ethanol (EtOH, VWR), GC grade 2-propanol (IPA, Sigma Aldrich, St. Louis, MO, US), HPLC grade acetonitrile (ACN, VWR) and type 1 water from a Milli-Q Ultrapure Water System (Millipore, Bedford, MA, US) or HPLC water (Chromasolv® plus for HPLC, Sigma Aldrich) were used as mobile phases (MP). MS grade formic acid (FA, Fluka), LC-MS grade ammonium acetate (Fluka), MS grade ammonium formate (AF, Fluka) and acetic acid (Fluka) were used as additives.

Stock solutions of sterols were made by dissolving standards in IPA. Standard solutions of native oxysterols were made by diluting stock solutions with an appropriate solvent while standard solutions for derivatized oxysterols were made from a working solution prepared by diluting stock solutions with IPA. Final concentration of stock solutions and working solutions together with vendors are listed in Table 1.

**Table 1: Sterols, concentration of stock solutions, concentration of working solutions together with vendors.**

Compound	Stock solution μg/mL (μM) in IPA	Working solution (nM) in IPA	Vendor
24S-hydroxycholesterol	10 (248)	1	Avanti Polar Lipids, Inc. Alabaster, AL, US
25-hydroxycholesterol	188 (467)	1	Sigma
27-hydroxycholesterol	150 (373)	1	Avanti Polar Lipids, Inc.
22S-hydroxycholesterol	188 (467)	1	Sigma
25-hydroxycholesterol 26,26,26,27,27,27 d <sub>6</sub>	6.12 (15)	1.5	CDN Isotopes, Quebec, Canada
Cholesterol 25,26,27 <sup>13</sup> C	224 (575)	6000	Sigma
Cholesterol	5000 (12931)		Sigma

### 2.1.1 Calibration solutions for quantification of derivatized oxysterols

Calibration solutions were prepared from 1 nM working solutions by dilution with IPA to cover the concentration range 14-136 pM for each oxysterol. The calibration solutions were subsequently subjected to sample preparation with derivatization as described in section 2.2.3.

### 2.1.2 Validation solutions

The validation solutions were used to assess the completeness of the derivatization procedure (D) in addition to matrix effects (M). Shh-L2 cancer cells were obtained from the Oslo University Hospital (Oslo, Norway). Cells were grown in Dulbecco's Modified Eagle



Medium (DMEM, from Sigma) with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin Solution (P/S) as described in [65]. Cells were lysed in 100  $\mu$ L 400 nM cholesterol  $^{13}\text{C}$  in EtOH. The validation sample solutions were spiked with standard solutions to give a concentration of 27, 54 and 108 pM of each oxysterol and were subsequently subjected to sample preparation with derivatization as described in section 2.2.3.

## 2.2 Samples

Samples were obtained by isolation of exosomes from cancer cells with either ultracentrifugation or a salting-out procedure using a commercial isolation kit as described below. The samples were subsequently subjected to sample preparation with derivatization is described in section 2.2.3.

### 2.2.1 Isolation of exosomes from cell culture medium with isolation kit

Preparation of cells and isolation of exosomes described in this section were performed by Hanne Røberg-Larsen at Oslo University Hospital by using the procedure described in [79]. Shh-L2 cell lines were grown in DMEM (Sigma or Gibco® (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, US)) with 10% FBS and 1% P/S. At 50% confluency, the cells were washed gently with pre-warmed (37°C) phosphate-buffered saline (PBS), and added DMEM media without FBS to harvest exosomes. After 24 or 72 hours cell culture medium was harvested and the cell medium was centrifuged at  $2\,000 \times g$  for 30 minutes to remove cells and cell debris. The supernatant containing cell-free culture medium was transferred to a new tube. Exosomes were isolated from 1, 3 or 14 mL cell culture medium using Total Exosome Isolation reagent (Invitrogen™ by Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, US) as described by producers. To 1, 3 and 14 mL cell culture media, 500  $\mu$ L, 1.5 mL and 7 mL reagent were added respectively, and the mixtures were vortexed until homogeneity. The samples were incubated overnight at 2-8°C. Subsequently the samples were centrifuged at  $10\,000 \times g$  at 4°C for 1 hour and the resulting supernatants

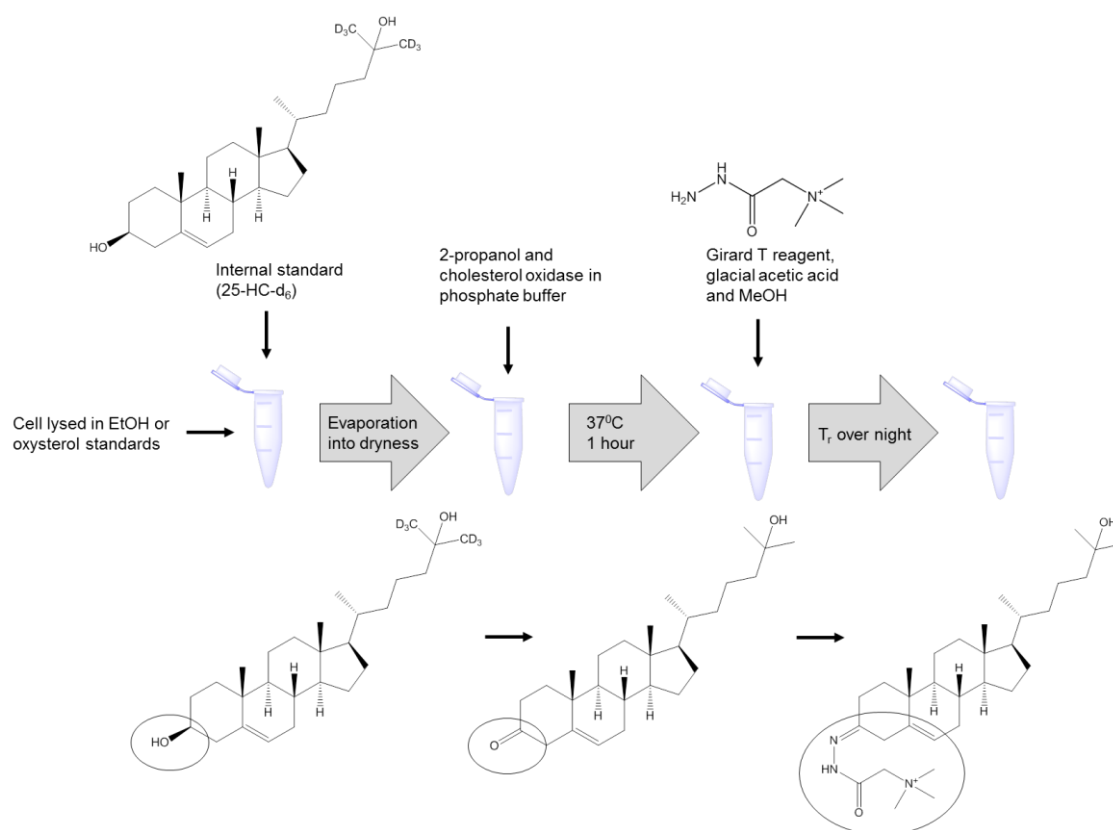
were aspirated and discarded. Finally, the pellets containing exosomes were re-suspended and lysed in 100  $\mu$ L 400 nM cholesterol  $^{13}\text{C}$  in EtOH. In addition, DMEM was subjected to the same isolation procedure without contact with cells (Sigma or Gibco®). Isolated exosomes were stored in the fridge (4°C) before sample preparation with Girard T reagent. The prepared samples were stored at 4°C and analyzed within a week.

### **2.2.2 Isolation of exosomes from cell culture media with ultra-centrifugation**

Preparation of cells and isolation of exosomes described in this section were performed by Hanne Røberg-Larsen and the author of this thesis at Oslo University hospital and Oslo Science Park with the procedure described in [80]. Shh-L2 cell lines were grown in DMEM medium (Gibco®) containing 10% FBS and 1% P/S until 70% confluency was reached. The medium was replaced with FBS free medium and incubated for 72 hours. Thereafter, as a supernatant, the medium was collected and transferred to 50 mL tubes and centrifuged at  $300 \times g$  for 10 minutes at 4°C to remove remaining free cells. The medium was again collected and transferred to a fresh centrifuge tube and centrifuged at  $2\,000 \times g$  for 20 minutes at 4°C to remove any cell debris. Further the medium supernatant was transferred to a tube suited for centrifugation at  $10\,000 \times g$  for 30 minutes at 4°C and the tube was marked and placed in the rotor with the mark is facing up. As this was a fixed angle rotor the pellet was found near the bottom of the tube and on the side of the tube facing up. The medium supernatant was again removed and transferred to a marked fresh tube and subjected to centrifugation at  $100\,000 \times g$  for 70 minutes at 4°C to pellet the exosomes. The medium supernatant was removed carefully and the remaining exosome pellet was washed by re-suspension in 1 mL PBS and centrifuged at  $100\,000 \times g$  for 70 minutes at 4°C. The PBS supernatant was removed and the remaining pellet was finally re-suspended and lysed in 300  $\mu$ L 400 nM cholesterol  $^{13}\text{C}$  in EtOH and stored at -80°C in the fridge before subjected to sample preparation. In addition, DMEM was subjected to the same isolation procedure without contact with cells (Gibco®). The prepared samples were stored in fridge at 4°C and analyzed within a week.

### 2.2.3 Derivatization of oxysterols with Girard T reagent

Derivatization of oxysterols with Girard T reagent was performed as described in [59, 65]. Standard solution and samples were mixed with internal standard (IS) and  $^{13}\text{C}$  cholesterol followed by evaporation to dryness on a SC110 Savant SpeedVac. Dry matter was re-dissolved in 20  $\mu\text{L}$  IPA and 200  $\mu\text{L}$  0.03 mg/mL cholesterol oxidase (Sigma) in 50 mM phosphate buffer (pH 7). The phosphate buffer was prepared by dissolving 679 mg  $\text{KH}_2\text{PO}_4$  (Fluka) in 100.0 mL type 1 water. The oxidation was carried out at  $37^\circ\text{C}$  for 1 hour using a Grant-Bio PHMT thermoshaker (Grant Instruments, Cambridge, UK) set to 300 rpm. After oxidation, 15 mg Girard T reagent (Sigma) dissolved in 500  $\mu\text{L}$  MeOH and 15  $\mu\text{L}$  glacial acetic acid (Merck) were added to each sample and standard solution before derivatization in room temperature ( $T_R$ ) in the dark overnight. A visual outline of the sample preparation is shown in Figure 10.



**Figure 10: Visual presentation of the sample preparation – derivatization of oxysterols with Girard T reagent. Adapted from [65].**

## **2.3 Instrumentation**

### **2.3.1 Direct infusion**

Solutions of 10 ng/ $\mu$ L native oxysterols were prepared by dilution with different mobile phase solvents and additives for direct infusion. Derivatized oxysterol solutions (1 ng/ $\mu$ L) were prepared for direct infusion as described in section 2.5.1. With the use of a syringe pump operated with a flow rate of 0.5  $\mu$ L/min, native or derivatized oxysterol solutions were directly infused into MS. The syringe was connected to 50  $\mu$ m ID polyimide coated fused silica tubing (Polymicro technologies, Phoenix, AZ) and a 30  $\mu$ m ID stainless steel emitter (Thermo Scientific, Waltham, MS, US) connected to an HESI or Nanospray flex ESI source on Q-Exactive™ hybrid quadrupole-Orbitrap or TSQ Quantiva™ triple quadrupole mass spectrometer, both from Thermo Scientific.

### **2.3.2 MicoLC system**

In the microLC system, 1  $\mu$ L of 800 pg/ $\mu$ L oxysterol was injected with an Agilent G1377A micro well autosampler (Agilent Technologies, Santa Clara, CA, US). An Agilent 1100 series solvent manager operated with a flow rate of 40  $\mu$ L/min delivering a mobile phase (MP A: 2.5 mM AF in H<sub>2</sub>O with 0.25% FA and MP B: 2.5 mM AF in MeOH with 0.25% FA) gradient from 70-100% B in 10 minutes. For separation a 1 mm ID  $\times$  150 mm ACE 3 C<sub>18</sub>-PFP column from Advanced chromatography technologies LTD (Aberdeen, Scotland, UK) was employed. The mobile phase was held at 100% B for 10 minutes after the gradient.

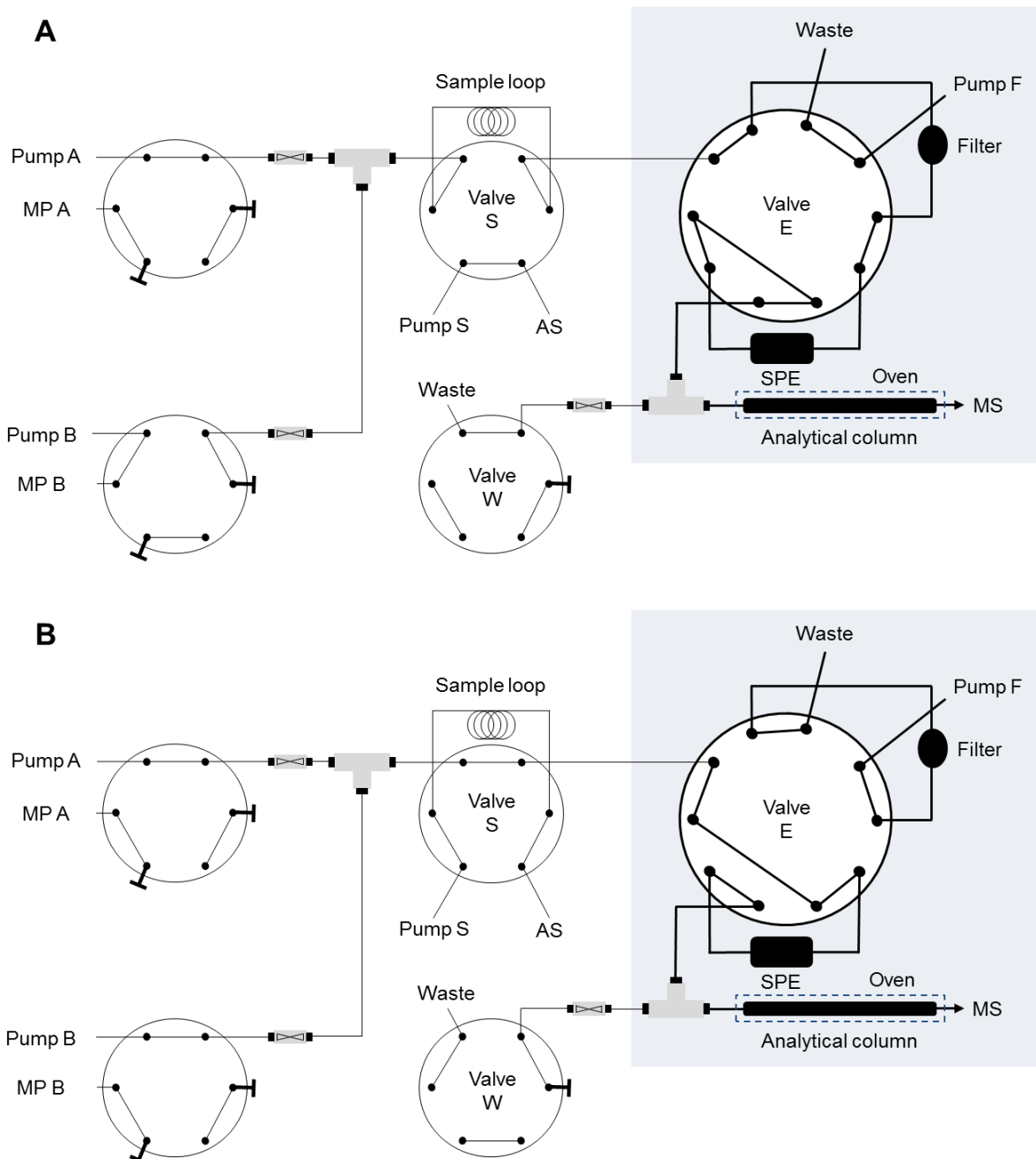
### **2.3.3 AFFL-SPE-nanoLC system I with on-line sample cleanup**

On-line sample cleanup was performed with an AFFL-SPE-nanoLC system as described in [65] and shown in Figure 6. Oxysterol standard solutions were injected (5  $\mu$ L) by an Agilent 1200 autosampler. An Agilent 1100 series pump (P1) was used as loading pump and filter back-flush pump delivering 0.1 % FA in H<sub>2</sub>O with a flow rate of 10  $\mu$ L/min. First, the sample went through a 1  $\mu$ m Valco (Huston, TX, US) stainless steel filter fitted in a Valco union.

Next, a reversed phase Hotsep Tracy Kromasil C<sub>8</sub> SPE column (0.3 mm ID × 5 mm, 5 μm, 300 Å) from G & T Septech (Ytre Enebakk, Norway) trapped the analytes (oxysterols). Other compounds not sufficiently hydrophobic to be trapped by the SPE column were flushed to waste. After an automatic switch of the 10-port two-position external valve from Waters (Milford, MA, US), an Agilent 1200 pump (P2) delivered a mobile phase (MP A: 2.5 mM AF in H<sub>2</sub>O with 0.25% FA and MP B: 2.5 mM AF in MeOH with 0.25% FA) gradient from 70-100% B in 10 minutes with a flow rate of 500 nL/min. Hence, the analytes were transferred from the SPE column to an ACE 3 C<sub>18</sub> analytical column (0.1 mm ID × 150 mm, 3 μm, 100 Å) for separation. The gradient was held at 100% B in 10 minutes to wash out other hydrophobic compounds. Simultaneously the loading pump cleaned the filter prior to the next injection. Chemstation software was used to control the pumps and switching valve.

#### **2.3.4 AFFL-SPE-nanoLC system II with on-line sample cleanup**

A Proxeon EASY nLC pump system from Bruker (Billerica, MA, US) was slightly modified by installing an external valve and a filter pump (Figure 11). To perform on-line sample clean-up, the autosampler of the Proxeon EASY nLC pump was set to inject 5 μL sample and 12 μL of FA/H<sub>2</sub>O/MeOH (0.1/80/20, v/v/v %) at max flow rate of 15 μL/min (or max 250 bar) were used to transfer the sample through a 1 μm stainless steel filter and loading of the sample onto a HotSep Tracy Kromasil C<sub>8</sub> column (0.3 mm ID × 5 mm, 5 μm, 300 Å) (G & T Septech). The SPE column was equilibrated with 4 μL 0.1% FA in H<sub>2</sub>O at a flow rate of 1 μL/min prior to each injection. After an automatic switch of the 10-port two-position external valve (Waters) a mobile phase (MP A 0.1% FA in H<sub>2</sub>O and MP B: 0.1% FA in MeOH) gradient from 75-90% B with a flow rate of 500 nL/min in 25 minutes transferred analytes (oxysterol derivatives) from the trap column to an ACE 3 C<sub>18</sub> or ACE 3 C<sub>18</sub>-PPF analytical column (0.1 mm ID × 150 mm, 3 μm, 100 Å) with subsequent separation of the analytes. The gradient was held at 90% B in 10 minutes to wash out other hydrophobic compounds. To dispose of unwanted particulates, a Knauer D-14163 pump (Berlin, Germany) was employed to back-flush the filter with 0.1% FA in H<sub>2</sub>O.

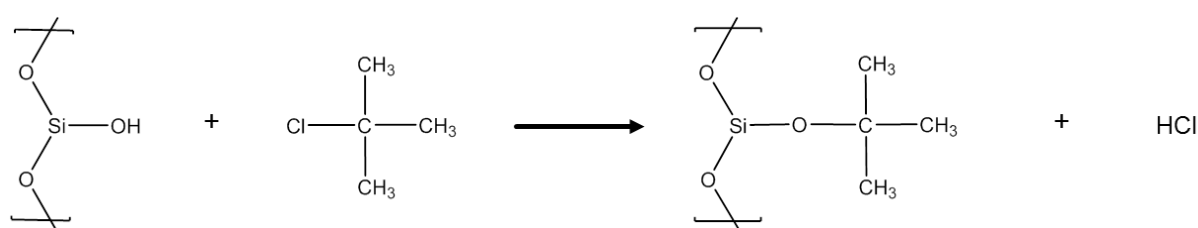


**Figure 11: AFFL-SPE performed with a 10-port two-position switching valve connected to a Proxeon EASY nLC-pump, shown in (A) load position where mobile phase A transfers the sample from sample loop through a stainless steel filter to remove particles and subsequent trapping of analytes onto the SPE column. Compounds that are not trapped on the SPE column are flushed to waste. After valve switching to (B) inject position mobile phase A and B are mixed and elute analytes from the SPE column onto the analytical column (placed in an oven) for separation prior to mass separation and detection. Simultaneously the filter is back-flushed by an external pump.**

## 2.4 Conditions specific for native oxysterol determination

### 2.4.1 Silanization of fused silica capillaries

Fused silica capillaries were silanized by flushing with 5% chlorotrimethylsilane (Sigma Aldrich) in n-heptane (Merck, Darmstadt, Germany) as described in [81]. The hydroxyl groups on the silica wall are replaced with trimethylsilane groups in the reaction shown in Figure 12.



**Figure 12: Silanization reaction of fused silica capillaries replacing hydroxyl groups with trimethylsilane groups.**

### 2.4.2 MS detection

A Q-Exactive™ hybrid quadrupole-Orbitrap equipped with a Nanospray flex ion source from Thermo Scientific was used in this work. The analytes were ionized using ESI in positive mode with a spray voltage of 1.5 kV and a capillary temperature of 320°C. Native oxysterols were monitored in SIM mode. Xcalibur Software (Thermo Scientific) was used for controlling the MS and for data collection.

## 2.5 Conditions specific for derivatized oxysterol determination

### 2.5.1 Off-line sample clean-up of standard solutions for direct infusion

Oxysterols were derivatized from a 1 ng/ $\mu$ L solution of oxysterols in IPA made from stock solutions. Excess of Girard T reagent was removed with a 100 mg C<sub>18</sub> Isolute (Biotage, Uppsala, Sweden) SPE column as described in [82]. The SPE column was conditioned with 1 mL MeOH followed by 1 mL type 1 water and a 2 mL mixture of MeOH and type 1 water (1+1). Subsequently 700  $\mu$ L derivatized oxysterol standard solution were applied and the eluate collected, diluted to 30% MeOH and re-applied. The SPE column was washed with 2 mL type 1 water and elution was performed with 1 mL MeOH followed by 1 mL chloroform (AnalaR®, VWR). The solvent was evaporated completely and the analytes were resolved in 700  $\mu$ L 0.1% FA in MeOH.

### 2.5.2 MS/MS detection

A TSQ Quantiva™ triple quadrupole mass spectrometer equipped with a Nanospray flex ion source (both from Thermo Scientific, Waltham, MS, US) was used in this work. The analytes were ionized by ESI in positive mode with a spray voltage of 1.25 kV and capillary temperature of 350°C. SRM mode with the transition  $m/z$  514.44  $\rightarrow$   $m/z$  455.36 was used to monitor the Girard T derivatives of 24-hydroxycholesterol (24S-HC-GT), 25-hydroxycholesterol (25-HC-GT), 27-hydroxycholesterol (27-HC-GT) and 22S-hydroxycholesterol (22S-HC-GT). The isotope labelled Girard T derivative of the internal standard and standard for cholesterol autoxidation were monitored using the respective transitions  $m/z$  520.40  $\rightarrow$   $m/z$  461.40 and  $m/z$  517.44  $\rightarrow$   $m/z$  458.36. The collision energy and RF lens (V) values were optimized separately for the ion transition of 25-HC-GT as a representative oxysterol derivative. The CID gas was set to 2 mTorr and the resolution on both Q1 and Q3 was operated at 0.2 full width half maximum (FWHM) and the dwell time was 600 ms. Data were collected using Xcalibur Software from Thermo Scientific.



## 2.6 Calculations

### 2.6.1 Quantification of derivatized oxysterols from calibration curve

The oxysterol concentration in samples was quantified by constructing a calibration curve based on linear regression ( $y = ax + b$ ) in Microsoft Excel. The calibration curve was based on the ratio of analyte area and internal standard area ( $y = A/A_{is}$ ) and the corresponding ratio of analyte concentration and internal standard concentration ( $x = C/C_{is}$ ). In SRM mode the extracted ion chromatogram (EIC) was smoothened before integration of peak areas with the use of Xcalibur Software.

$$C = \frac{\frac{A}{A_{IS}} - b}{a} \cdot C_{IS}$$

$$a = slope$$

$$b = intercept$$

### 2.6.2 Completeness of the derivatization reaction and matrix effects

The completeness of derivatization reaction (D) and matrix effects (M) were calculated based on linearity curves from cell samples compared with standard solutions at three concentration levels (27pM, 54pM and 108 pM,  $n = 3$ ) and by using the formula below.

$$Recovery = \frac{a_1}{a_2} \times 100\%$$

$$a_1 = slope_{spiked\ samples}$$

$$a_2 = slope_{standard\ solution}$$

## 3 Results and discussion

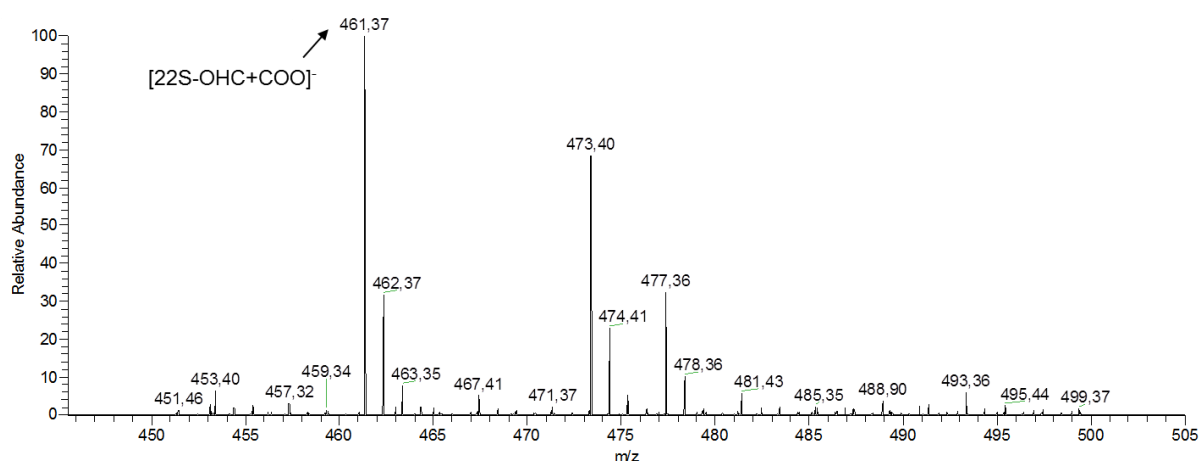
As oxysterols investigated in this study (24S-OHC, 25-OHC, 27-OHC and 22S-OHC) are neutral species, they are difficult to ionize efficiently with ESI. Insufficient ionization of molecules using ESI can lead to reduced sensitivity of the method. A solution can be to derivatize oxysterols into charged compounds. However, this requires time-consuming sample preparation. As an alternative, formation of charged adducts between oxysterols and mobile phase additives may be a simpler approach for detecting oxysterols with ESI-MS. The approaches are presented and discussed in the following sections and published [82].

### 3.1 Determination of native oxysterols

#### 3.1.1 Provoking adduct formation to improve ESI-MS detection of native oxysterols

With the objective to quantify oxysterols using nanoLC-ESI-MS, a first step in method development is to obtain (repeatable) signal intensities in MS and preferably a main fragment in MS/MS for identification purposes. Unrepeatable signal intensities can lead to poor accuracy and precision of the analytical method (especially if dedicated labelled internal standard are not employed). It has been reported that native oxysterols form adducts with ammonium acetate [63]. Therefore, solutions of oxysterols in 10 mM ammonium acetate in either MeOH, IPA or ACN were analyzed to see if the ions in the solution would form ESI-MS detectable adduct species (e.g. between oxysterols and  $\text{NH}_4^+$  from an ammonium acetate containing solution). The solutions were infused directly to the MS with either positive or negative ESI (using either the HESI or the Nanospray flex ion source). Adducts between acetate and a representative oxysterol (22S-OHC) were observed in negative ESI-MS mode ( $[\text{22S-OHC}+\text{COO}]^-$ ,  $m/z$  461.37) using the Nanospray flex ion source, as shown in Figure 13. No adduct ions were observed in positive ESI (with neither the Quantiva™ nor the Q-Exactive™ Orbitrap instruments equipped with the HESI or the Nanospray flex ESI source) in contrast to that described by McDonald et al. (who used an AB Sciex 4000 QTrap instrument) [63].

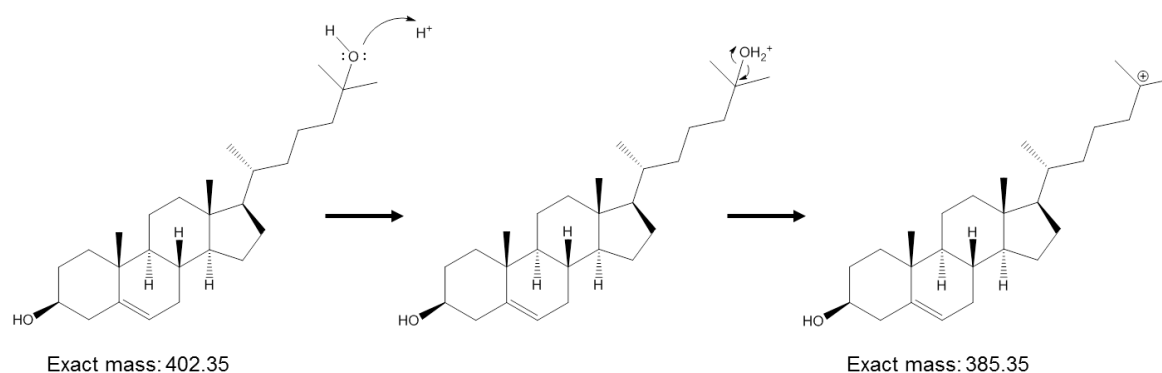
However, the signal intensity of the negative adducts observed varied significantly from day to day ( $n = 2$ ). This did not improve when varying other parameters e.g., ESI voltages and capillary temperature (Figure 7), so the acetate-oxysterol adduct approach was not further considered. None of the solutions tested produced improved adduct signal or repeatable signal intensity. Hence, detection of adducts between oxysterol and mobile phase ions was considered unfeasible, with the instrumentation available. More information regarding attempts to achieve adduct signal or repeatable signal intensity of adducts on Q-Exactive™ Orbitrap can be found in Appendix, section 6.1.



**Figure 13:** MS spectrum of acetate adducts of 10 ng/μL 22S-OHC ( $m/z$  461.37, [22S-OHC+COO]<sup>-</sup>) with optimized spray voltage (1.0 kV) and capillary temperature (200°C). The ion signal intensity was not repeatable from day to day ( $n = 2$ ).

### 3.1.2 Ionization of native oxysterols without adduct formation

Others have reported ESI-MS visibility of oxysterols that has undergone loss of one or two water molecules in the ESI source [58, 61, 83]. When voltage is applied to the ESI capillary tip a hydroxyl group in the oxysterol molecule can take up a proton from the mobile phase. This generates an oxonium ion and subsequent loss of water leaves the oxysterol with a positive charge (Figure 14).



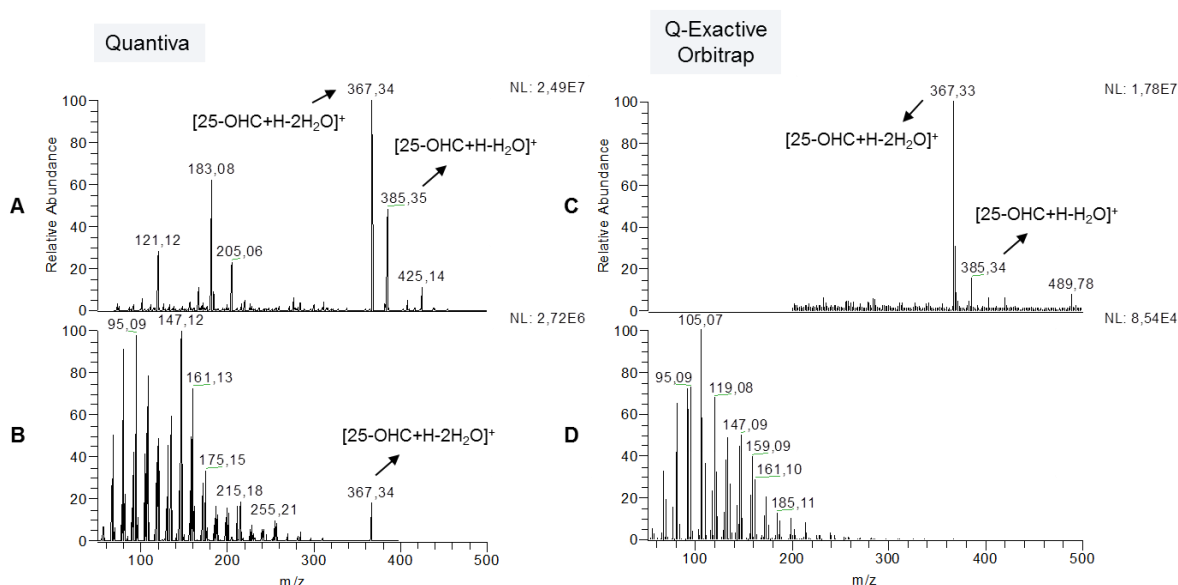
**Figure 14: Proposed mechanism for oxysterols that undergoes loss of one water molecule. When voltage is applied to the ESI capillary tip a hydroxyl group in the oxysterol molecule can take up a proton from the mobile phase generating an oxonium ion and subsequently loose water leaving the oxysterol with a positive charge.**

By diluting e.g. 25-OHC in 0.1% FA in MeOH, loss of water ions ( $[M+H-H_2O]^+$  and  $[M+H-2H_2O]^+$ ,  $m/z$  385.35 and  $m/z$  367.34) were observed in MS with both the Quantiva™ and Q-Exactive™ Orbitrap instruments. The signal intensity of these species was however not repeatable from day to day ( $n = 2$ ). Different solutions (e.g. 0.1-0.25% FA in MeOH or IPA, 2.5 mM AF in MeOH or IPA, AF/FA in MeOH or IPA and 10 mM AF in 70% ACN) listed in Table 2 were investigated to achieve repeatable signal intensities of the loss-of-water-ions. By using a combination of FA (0.25%) and AF (2.5 mM) in the solvent the ion signals remained relatively stable with repeatable signal intensities with both MS instruments (tested 2 days), see Figure 15 A and C.

**Table 2: Solutions used to investigate ionization of native oxysterols in ESI-MS with the use of either the Nanospray flex ESI or the ESI (HESI) ion source. Table modified from: [82].**

Solutions	Results and comments
0.1-0.25% FA in MeOH or IPA	Loss of one or two water molecules. Large variation in signal intensity.
2.5 mM AF in MeOH or IPA	Loss of one or two water molecules. Large variation in signal intensity.
AF/FA in MeOH or IPA	Loss of one or two water molecules. Stable signal intensities.
10 mM AF in 70% ACN	No signal.

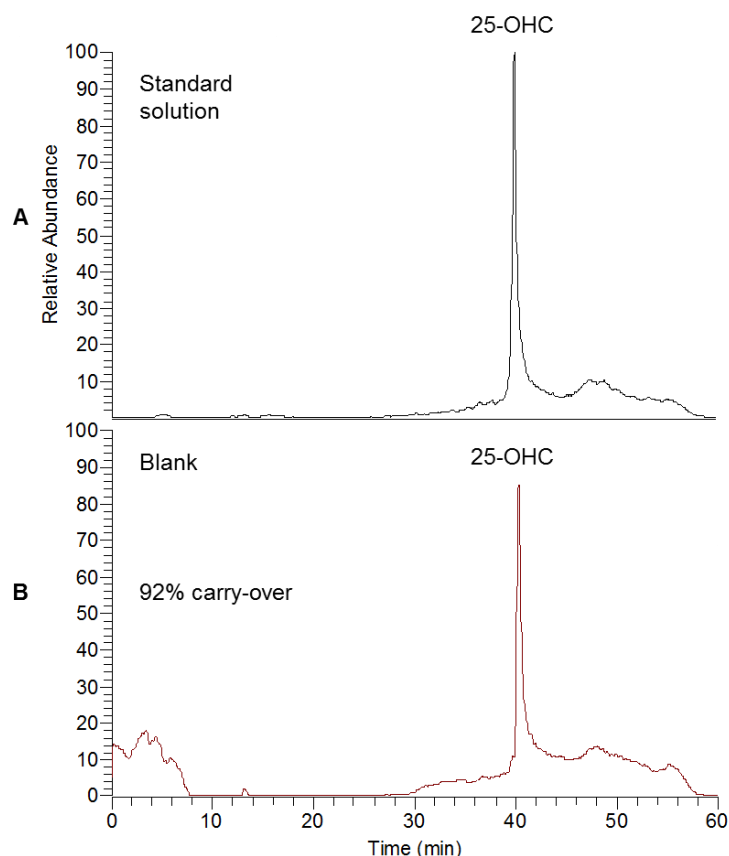
In attempt to achieve optimal MS/MS conditions, fragmentation as function of collision energies in MS/MS was investigated. However, the resulting MS/MS spectrum was dominated by low mass area clusters with both the Quantiva™ (Figure 15 B) and the Q-Exactive™ Orbitrap instrument (Figure 15 D), probably due to lack of charged centers in the molecule [82], which can be associated with undefined MS/MS spectra. As a consequence, finding interpretable and repeatable MS/MS transitions was not possible, severely limiting the utility of MS/MS. In contrast, derivatization approaches are associated with clear and repeatable MS/MS transitions [59, 65], enabling a high degree of method selectivity.



**Figure 15:** (A) MS spectrum of 10 ng/μL 25-OHC in 2.5 mM AF in MeOH with 0.25% FA ( $m/z$  385.35 ( $[M+H-H_2O]^+$ ) and  $m/z$  367.34 ( $[M+H-2H_2O]^+$ )) and (B) MS/MS spectrum of  $m/z$  367.34 ( $[M+H-2H_2O]^+$ ) showing clustering in the low mass area when fragmenting the molecular ion using the Quantiva™ instrument. (C) MS spectrum of 10 ng/μL 25-OHC in 2.5 mM AF in MeOH with 0.25% FA ( $m/z$  385.34 ( $[M+H-H_2O]^+$ ) and  $m/z$  367.33 ( $[M+H-2H_2O]^+$ )) and (D) MS/MS spectrum of  $m/z$  367.33 ( $[M+H-2H_2O]^+$ ) obtained with a HCD energy of 30, showing the same tendency of clustering in the low mass area using a Q-Exactive™ Orbitrap instrument. Figure modified from [82].

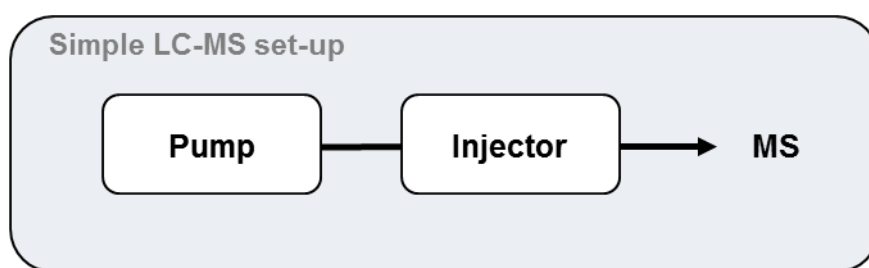
### 3.1.3 Carry-over and adsorption issues of native oxysterols in nanoLC

Although MS/MS could not be employed in analysis of native oxysterols, it could still be possible to achieve acceptable specificity via stable retention times and high resolution mass spectrometry. The performance of nanoLC was hence investigated for the oxysterols. The chromatography was considered acceptable as the eluting peaks had a peak width of ~1 minute. Gradually (after 5-20 injections), large and persistent carry-over (> 50%) was observed (Figure 16).



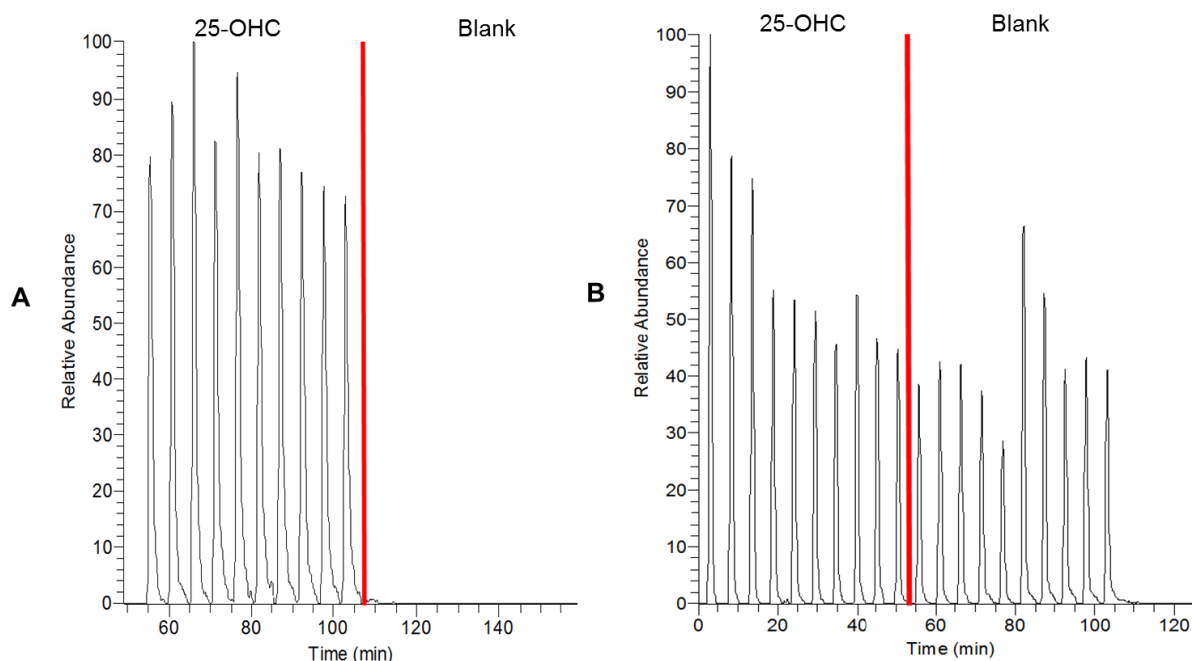
**Figure 16: Chromatogram of (A) 800 pg/ $\mu$ L 25-OHC and (B) MeOH, showing ~90% carry-over of 25-OHC from the AFFL-SPE-nanoLC system. Analysis was performed on an ACE 3 C<sub>18</sub> (0.1 mm ID  $\times$  150 mm) analytical column with gradient mobile phase (MP A: 2.5 mM AF in H<sub>2</sub>O with 0.25% FA and MP B: 2.5 mM AF in MeOH with 0.25% FA) 70-100% B in 15 minutes at 40  $\mu$ L/min. The injection volume was 6  $\mu$ L. Signals are normalized with regard to A.**

To locate sources of carry-over in the AFFL-SPE-nanoLC-MS system, the system was stripped down step by step to a simple set-up consisting of only a pump, manual injector and the MS instrument, all coupled together with fused silica tubing (Figure 17). Still, a high degree of carry-over was observed. Therefore, the origin of carry-over effects was attributed (at least in part) to fused silica capillaries. It was speculated that the silanol groups in fused silica adsorbed the oxysterols to the walls (possibly through hydrogen bonding), creating a hydrophobic layer that oxysterols from subsequent injections would adsorb to.



**Figure 17: Outline of the simple LC-MS set-up consisting of a pump, manual injector and the MS instrument all coupled together with fused silica tubing. The set-up was employed to investigate carry-over sources.**

As an alternative to fused silica capillaries, polyether ether ketone (PEEK) tubing was considered, but these are associated with swelling/clogging issues in nanoLC systems [82] and were not used. As no other suitable tubing is available for nanoLC (to author's knowledge), all capillaries exposed to oxysterols were silanized, exchanging hydroxyl groups on the silica surface with trimethylsilane groups (Figure 12). Using silanized tubing to connect instrumental parts in the simple set-up was successful. With silanized capillaries no carry-over was observed (Figure 18 A) while with untreated fused silica capillaries the carry-over effect remained (Figure 18 B).



**Figure 18:** Carry-over investigation by 10 injections of 10 pg/ $\mu$ L 25-OHC (2  $\mu$ L injected) followed by 10 injections of 2.5 mM AF in IPA on (A) silanized fused silica tubing (50  $\mu$ m ID) and (B) fused silica tubing (50  $\mu$ m ID). Analysis was performed using a simple LC-MS set-up. Mobile phase was 2.5 mM AF in IPA delivered with a flow rate of 4  $\mu$ L/min. Figure modified from [82].

Since no carry-over was observed when silanized tubing was utilized in the simple LC-MS set-up, a representative oxysterol standard solution (25-OHC) was injected into the AFFL-SPE-nanoLC system (Figure 6) coupled together with silanized tubing. Acidic or buffered loading and mobile phase solutions employed (Table 3) in the system should have the ability to both trap/retain and elute the oxysterol from the pre-column and analytical column. However, the combination of solutions investigated for this purpose (e.g. 2.5 mM AF in H<sub>2</sub>O/MeOH (90/10, v/v/ %) with 0.25% FA as loading phase and 2.5 mM AF in MeOH with 0.25 % FA as mobile phase solution), either caused breakthrough or carry-over in the system, hence these solutions were considered inappropriate.



**Table 3: Acidic and buffered solutions used as loading and eluting mobile phase for LC investigations.**  
Table modified from: [82].

<b>Loading mobile phase</b>	<b>Eluting mobile phase</b>	<b>Results/comments</b>
0.1% FA in H <sub>2</sub> O	0.1% FA in MeOH or IPA	Oxysterols trapped, large carry-over
0.1% FA in MeOH (< 10%)	0.1% FA in MeOH or IPA	Oxysterols trapped, large carry-over
0.1% FA in MeOH (> 10%)	0.1% FA in MeOH or IPA	No retention, carry-over not examined
0.1% FA in EtOH (> 5%)	0.1% FA in EtOH	No retention, carry-over not examined
AF/FA in H <sub>2</sub> O	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over
AF/FA in MeOH (< 10%)	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over
AF/FA in MeOH (> 10%)	AF/FA in MeOH or IPA	No retention, carry-over not examined
	0.1% FA in MeOH (< 65%)	Solubility issues, poor peak shape
	0.1% FA in MeOH (> 65%)	Large carry-over
	0.1% FA in IPA	Large carry-over
	AF/FA in MeOH	Large carry-over
	AF/FA in IPA	Large carry-over

In order to trace additional origins of the carry-over, instrumental parts (e.g. the silica based pre-column, injection needle and loop, spray needle and the 10-port valve used to perform column switching) were exchanged or removed from the system prior to a new injection. Possible sources of carry-over were not localized and the efforts made are presented as an overview in Table 7 in Appendix, section 6.2. Furthermore, possible carry-over was also examined in a microLC system as others determining native oxysterols using microLC or larger scale systems do not report carry-over issues [58, 61, 63]. With larger column/tubing dimensions, carry-over was not observed, suggesting that this may be a characteristic of nanoLC. This can possibly be due to a larger surface to volume ratio in the narrow tubing and connections employed in nanoLC. For more details on the microLC experiment, see Appendix section 6.2.1.

Thus, developing a sensitive method for determination of native oxysterols using nanoLC was considered unattainable considering of the large carry-over effects observed in RP conditions. In order to maintain the oxysterols in a soluble form and to preserve method sensitivity with the use of nanoLC (advantageous when working with biological samples with low concentration of oxysterols), a derivatization approach can be recommended when RP-LC is used as separation principle [65]. However, as these carry-over effects are not observed in a microLC system, analysis of underivatized oxysterols is possible in a larger scale system.

## **3.2 Determination of derivatized oxysterols**

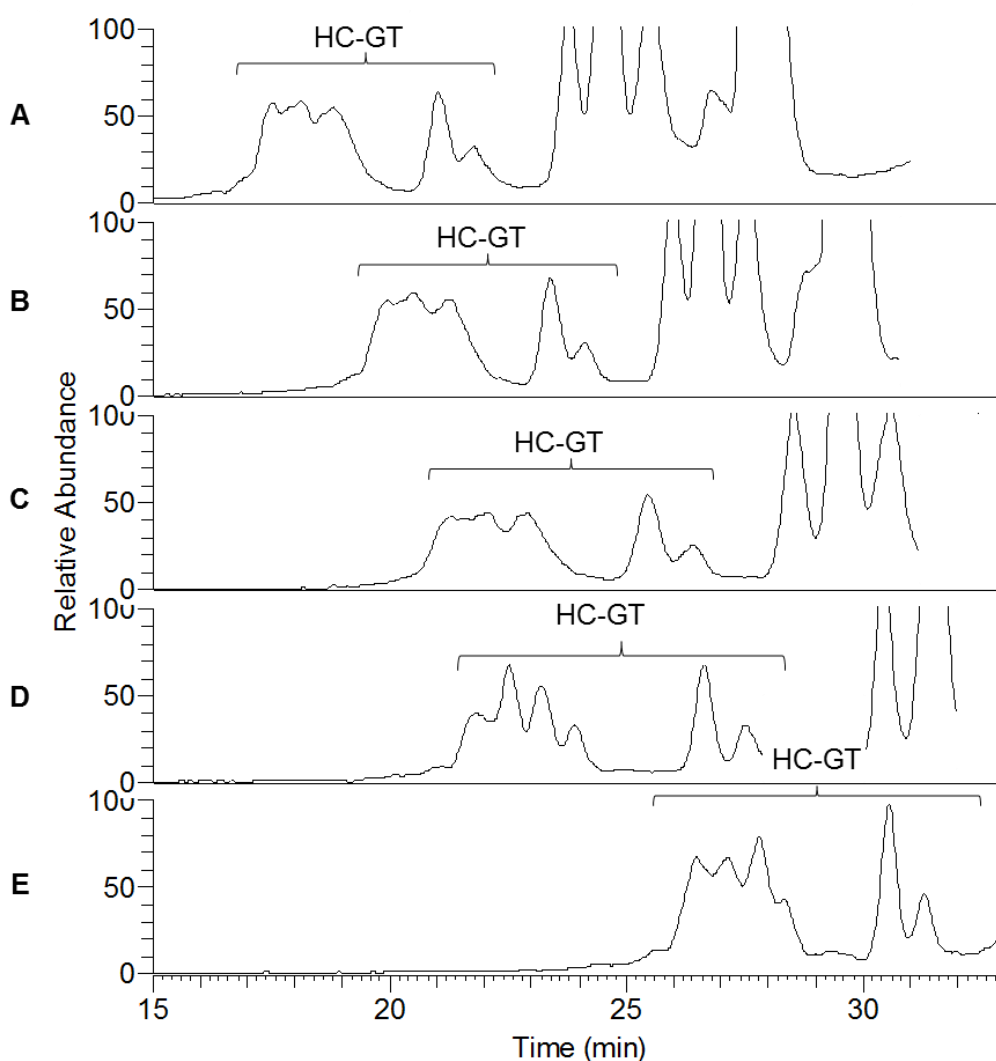
As it was not attainable to develop a sensitive method for determination of native oxysterols, using nanoLC, our established method [65] using derivatization was modified to provide better separation of the oxysterol isomers. The modified method was intended to be used for determination of Girard T derivatized oxysterols in exosomes samples.

### **3.2.1 Separation of derivatized oxysterol isomers**

The derivatized oxysterol isomers 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC GT have similar LC-MS properties and their monoisotopic and main fragment masses are the same. Therefore, high resolution chromatographic separation is needed in order to identify individual isomers. Commercially available ACE columns (0.1 mm ID × 150 mm) with different functionalities as C<sub>18</sub> and C<sub>18</sub>-PFP was investigated as studies has shown that the ACE columns has an appropriate carbon load (15.5 %) suited for separation of oxysterol isomers [59, 65, 84].

Initially a C<sub>18</sub> column and isocratic mobile phase consisting of FA/H<sub>2</sub>O/MeOH in ratios from 0.1/30/70 (v/v/v %) to 0.1/5/95 (v/v/v %) was examined using the AFFL-SPE-nanoLC system for separation of (14-136 pM) oxysterol derivatives. With the Proxeon EASY nLC pump system, separation of the analytes with isocratic elution was not achievable. In a default set-up (i.e. 100% aqueous loading solvent) the analytical column is inevitably exposed to relatively large amount of water, causing solubility issues (oxysterols are hydrophobic) before the organic separation mobile phase reaches the nanoLC column. This resulted in poor chromatographic conditions and hence poor resolution and peak shapes. Using a higher organic content in the aqueous mobile phase solvent resulted in poor recovery. It was speculated that a gradual transition from highly aqueous to highly organic solvents (i.e. solvent gradients) to carefully dissolve the trapped analytes prior to elution onto the LC column would provide improved chromatographic conditions for the hydrophobic analytes.

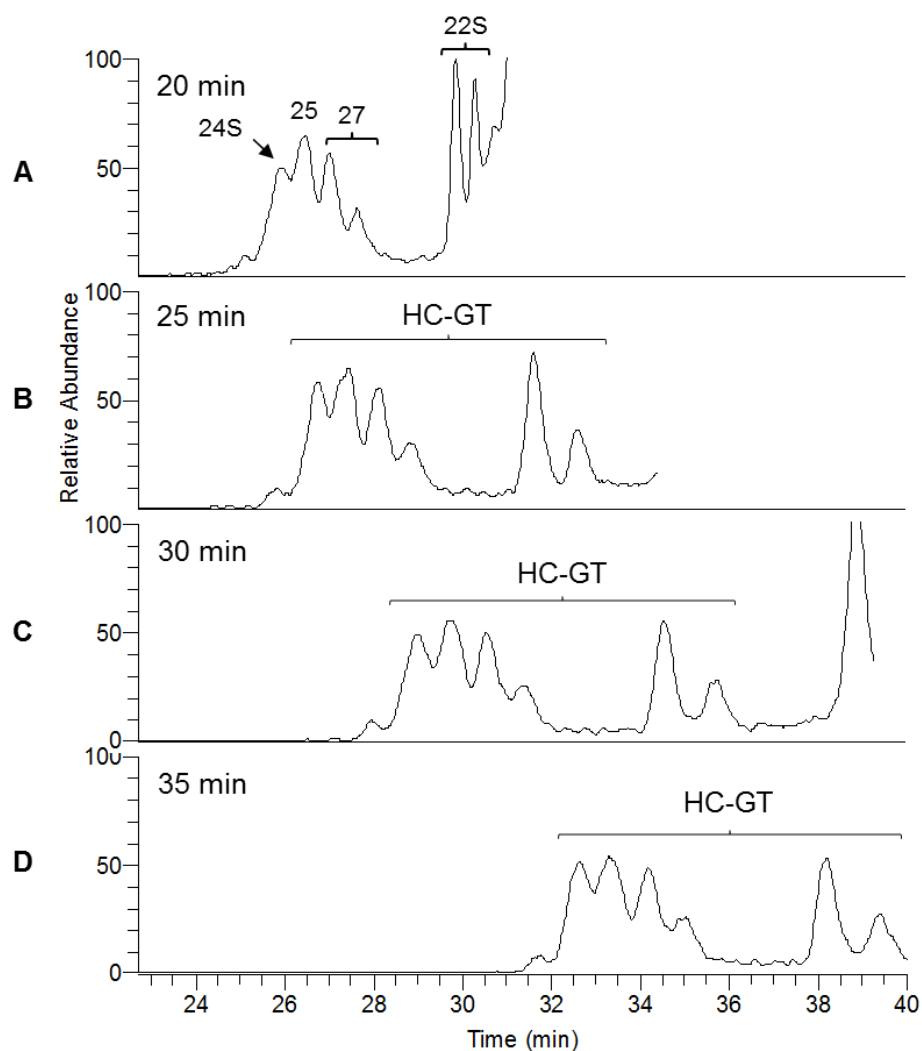
Several gradients were examined and the most promising results (i.e. best peak resolution) were obtained using a gradient mobile phase (MP A: 0.1% FA in H<sub>2</sub>O and MP B: 0.1% FA in MeOH) elution from 75-90% B in 25 minutes using a C<sub>18</sub> column. A decrease in the organic content to 70% B or 65% B in starting gradient conditions resulted in a higher degree of co-elution of the species possibly due to solubility issues that cause band broadening (Figure 19).



**Figure 19: EIC of derivatized oxysterols ( $m/z$  514.44  $\rightarrow$  455.36) in an 81 pM standard solution analyzed using the AFFL-SPE-nanoLC system and the Quantiva<sup>TM</sup> MS instrument. Analysis was performed on an ACE C<sub>18</sub> column with gradient mobile phase (MP A: 0.1% FA in H<sub>2</sub>O and MP B: 0.1% FA in MeOH) from (A) 75-95% B, (B) 70-95% B, (C) 70-90% B, (D) 75-90% B and (E) 65-90% B in 25 minutes at 500 nL/min. Injection volume was 5  $\mu$ L and sample clean-up was performed on-line. The filter back-flush solution consisted of 0.1% FA in H<sub>2</sub>O and the loading mobile phase consisted of FA/H<sub>2</sub>O/MeOH (0.1/70/30, v/v/v %). Loading volume was set to 12  $\mu$ L with a max flow rate of 15  $\mu$ L/min/250 bar and the filter back-flush with a flow rate of 100  $\mu$ L/min. Signal intensities are normalized with regard to E.**

Different gradient lengths i.e. 20, 25, 30 and 35 minutes were also examined (Figure 20). Going from 20 to 25 minutes improved the resolution as well as 22S-HC-GT was allowed to elute before the unidentified peaks with higher intensity. The apparent resolution was considered to be the same with a gradient length of 25, 30 and 35 minutes. Therefore, the

shortest gradient time with sufficient resolution, 25 minutes, was chosen to reduce the time of analysis.



**Figure 20: EIC of derivatized oxysterols ( $m/z$  514.44  $\rightarrow$  455.36) in an 81 pM standard solution** Analysis was performed on an ACE  $C_{18}$  column with gradient mobile phase (MP A: 0.1% FA in  $H_2O$  and MP B: 0.1% FA in MeOH) elution from 75-90% B in (A ) 20 minutes, (B) 25 minutes, (C) 30 minutes and (D) 35 minutes with a flow rate of 500 nL/min. Other chromatographic conditions were as described in Figure 19. Signals are normalized with regard to A.

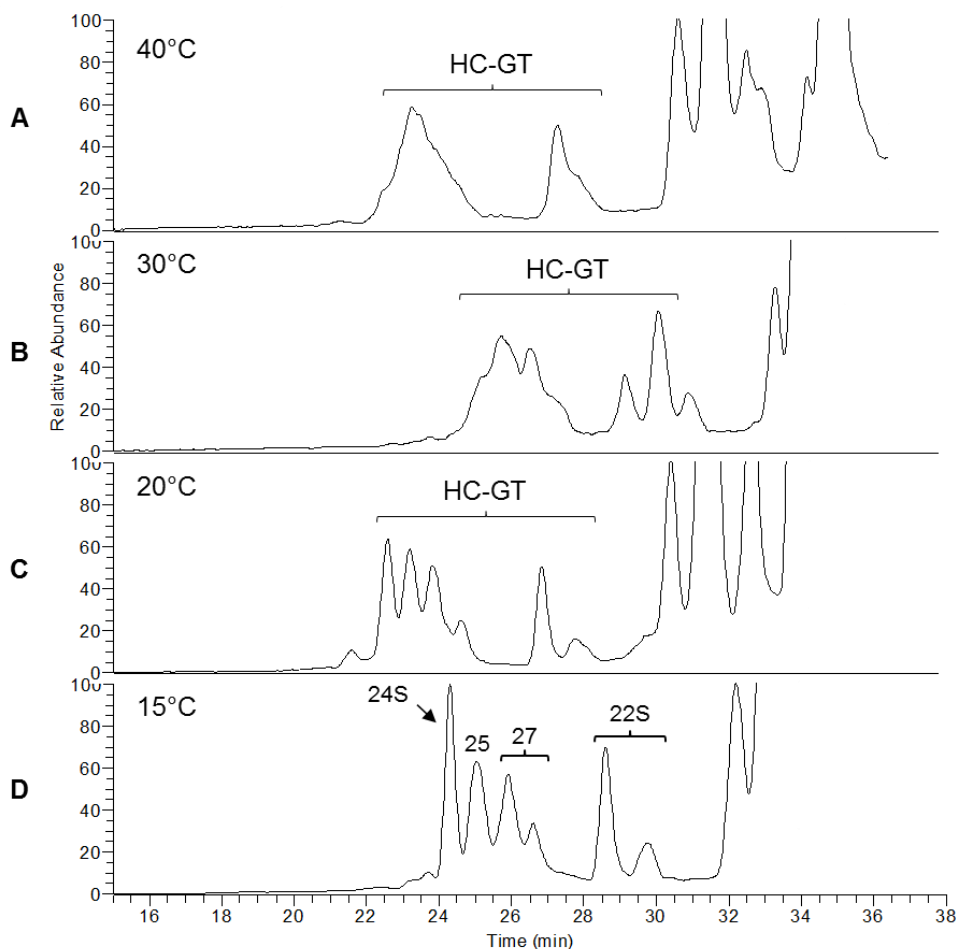
Gradient separations with a  $C_{18}$ -PFP column did not enable improved analyte resolution, as seen in Figure 36 in Appendix. To enhance the  $C_{18}$  column efficiency by increasing the

column length, a combination of two C<sub>18</sub> columns was also investigated. However, the resolution was poor, probably due to more dead volumes in couplings between the columns. More details from this experiment are presented in section 6.4 in Appendix. Therefore, a single C<sub>18</sub> column with the described gradient was further employed, and temperature as a separation parameter was subsequently explored.

### 3.2.2 Effect of temperature

To further improve the resolution, temperature as a separation parameter was investigated. This was performed by analysis of standard solutions containing 81 pM 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT separated with gradient elution from 75-90% B in 25 minutes and with a column temperature of 40°C, 30°C, 20°C or 15°C. The flow rate was set to 500 nL/min for all analyses. A decrease in temperature provided enhanced resolution while elevated temperatures resulted in co-elution of the oxysterol derivatives, as seen from Figure 21. However, the retention time of the analytes seems to be nearly unaffected by changes in temperature as the analytes eluted in the same time range regardless of the temperatures investigated. With a further decrease to 10°C the pressure limit (300 bars) of the pump was exceeded, hence 15°C was chosen as column temperature. With a temperature of 15°C in the column, the flow rate could be 400 nL/min, 450 nL/min or 550 nL/min without affecting the plate height (H). Still, a 500 nL/min flow rate was chosen as higher flow rate increased the backpressure close to 300 bars, which can decrease the ruggedness of the method and the column lifetime.

In Figure 21, peak splitting of 27-HC-GT and 22S-HC-GT is observed. This can be explained by syn and anti-formations from the derivatization reaction and two peaks appear as their chromatographic selectivity is slightly different [59, 65].

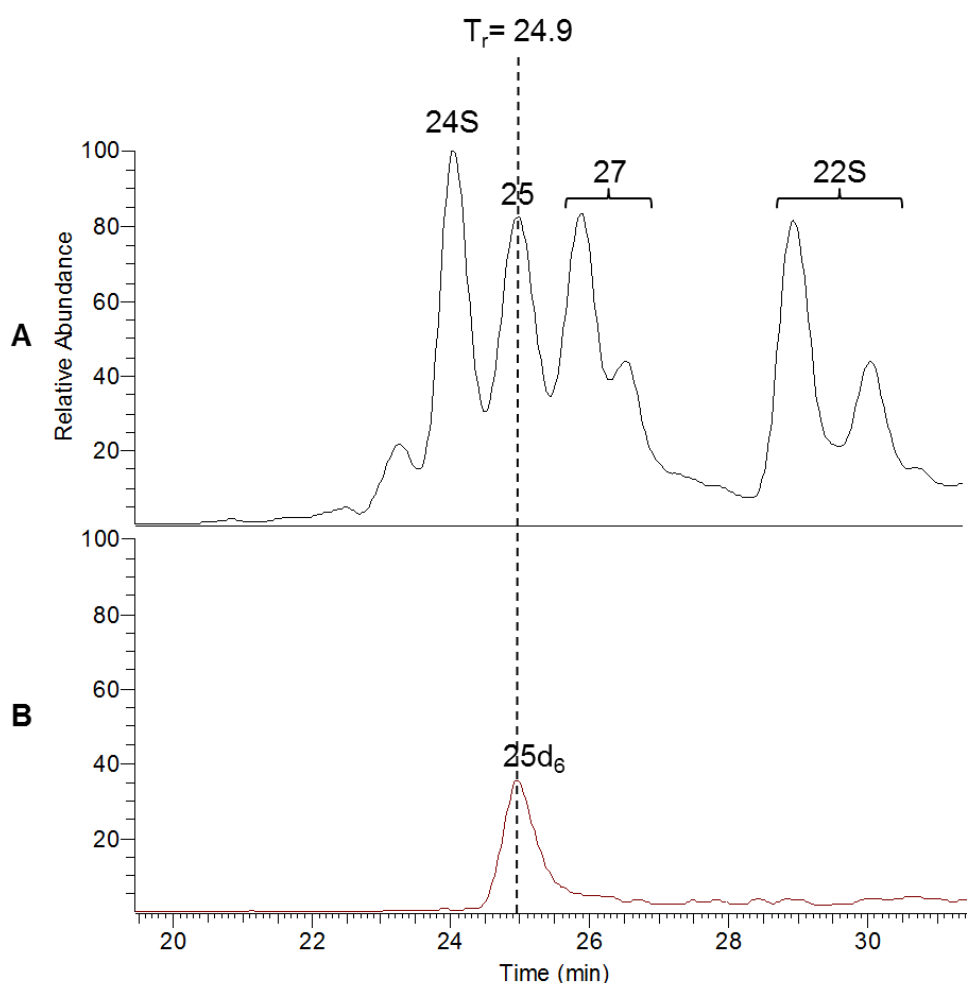


**Figure 21:** EIC of derivatized oxysterols 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT ( $m/z$  514.44  $\rightarrow$  455.36) in an 81 pM standard solution showing the effect of (A) 40°C, (B) 30°C, (C) 20°C and (D) 15°C in the analytical column. Best apparent resolution and separation were achieved with a column temperature of 15°C. Other chromatographic conditions were as described in Figure 19. Signals are normalized with regard to D.

### 3.2.3 Internal standard

The completeness of the derivatization reaction and the ionization efficiency in MS may vary. Therefore, an internal standard is employed to correct for these factors among others. Ideally, the internal standard should behave in the same way as the analyte during sample preparation and analysis. Hence, the analyte and the internal standard should have shared characteristics. The use of MS gives the opportunity to use an isotope labelled oxysterol as an internal standard and 25 hydroxycholesterol 26,26,26,27,27,27  $d_6$  which was employed in the

established method [65] was also used in this study. Chromatograms of the analytes together with the internal standard are shown in Figure 22 A and B, respectively. As expected, the internal standard has the same retention time ( $T_r$ ) as 25-HC-GT. Preferably, deuterated standards for all analytes should be implemented in the method. However, due to economic considerations and analytes eluting closely together, one deuterated standard was considered sufficient.

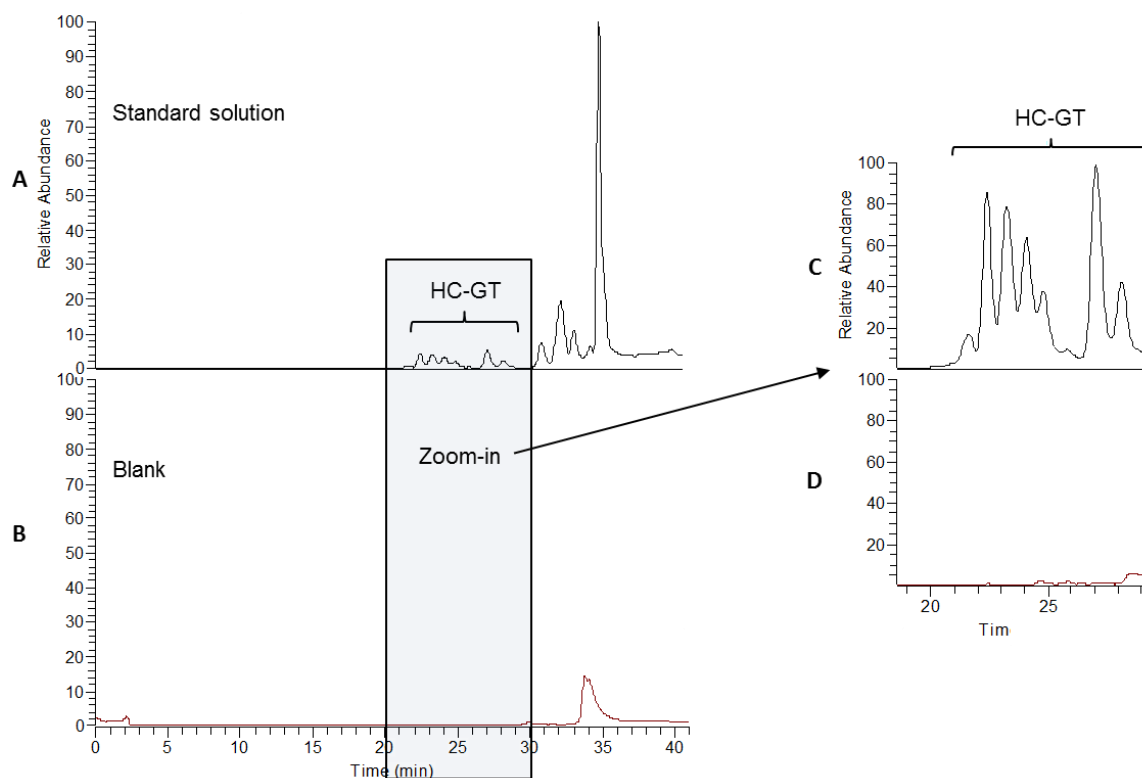


**Figure 22:** EIC of (A) 136 pM derivatized 24S-HC-GT ( $T_r = 24.9$ ), 25-HC-GT ( $T_r = 24$ ), 27-HC-GT ( $T_r = 25.9$  and  $26.5$ ), and 22S-HC-GT ( $T_r = 28.9$  and  $30$ ), ( $m/z$  514.44  $\rightarrow$  455.36) and (B) 50 pM internal standard (25d<sub>6</sub>-HC-GT ( $T_r = 24.9$ ),  $m/z$  520.40  $\rightarrow$  461.40) in a standard solution. Separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C. Other chromatographic conditions were as described in Figure 19. Signal intensities are normalized with regard to A.



### 3.2.4 Examination of carry-over in the AFFL-SPE-nanoLC system

With the use of column switching techniques, carry-over between samples can occur. This affects accuracy and precision of the method. Investigation of carry-over effects was performed by injecting 5  $\mu$ L MeOH after analysis of standard solutions containing 14-136 pM 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT. With the use of the AFFL-SPE-nanoLC system with gradient elution from 75-90% B in 25 minutes and a column temperature of 15°C, minimal carry-over was found, below 1% (Figure 23). Carry-over was also investigated after injection of samples with the same results (data not shown).



**Figure 23:** EIC of derivatized oxysterols ( $m/z$  514.44  $\rightarrow$  455.36) in (A) 136 pM standard solution following a (B) MeOH (blank) injection showing minimal carry-over effects, below 1%. Separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C. Other chromatographic conditions were as described in Figure 19. Signal intensities are normalized with regard to A and C respectively.

### 3.2.5 SPE breakthrough

Breakthrough of analytes from the SPE can occur both if the loading solvent and the sample solvent has high elution strength. The solvents used in the chromatographic system should be able to trap and elute the analytes sufficiently in addition to maintain the analytes in a soluble form. From earlier experiments, it was found that 30% MeOH in the loading phase was sufficient for trapping the Girard T derivatized oxysterols on the SPE in a microLC system (standard solution contains 67% MeOH after derivatization) [84]. However, after ~100 injections, peak areas declined, indicating the beginning of breakthrough issues. The sudden breakthrough can be caused by gradual modifications of the SPE column material by other hydrophobic compounds in the samples and standard solutions. For instance cholesterol, which is present in standard solutions and samples, and has been reported to change column material properties [50]. To solve the breakthrough issue, an SPE wash with ACN was implemented in the method. After each injection a large volume of ACN (10 times  $\times$  SPE column volume) was injected. In addition, the organic content of the loading solvent was reduced to 20% MeOH. As a result, breakthrough issues were no longer observed.

### 3.2.6 Final separation conditions

Sufficient resolution ( $R_s \sim 1-1.3$ ) was achieved with the  $C_{18}$  column by using a gradient mobile phase elution (MP A: 0.1 % FA in  $H_2O$  and MP B: 0.1 % FA in MeOH). The gradient was 75-90% B in 25 minutes with a flow rate of 500 nL/min and a column temperature of 15°C. Sample clean-up was performed on-line with the AFFL-SPE-nanoLC system. The sample (5  $\mu$ L) was loaded onto the SPE column with 12  $\mu$ L loading mobile phase (FA/ $H_2O$ /MeOH (0.1/80/20, v/v/v %)) and with max flow rate or max pressure of 15  $\mu$ L/min and 250 bar respectively. The filter was back-flushed with 0.1% FA in  $H_2O$  with a flow rate of 100  $\mu$ L/min. These conditions allow sample clean-up and applicable separation of 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT within ~30 minutes as seen in Figure 22. Total analysis time including wash of SPE and re-equilibration of column was 60 minutes.

### **3.2.7 Isolation of exosomes**

In order to find a suitable procedure to study the possible connection between oxysterols and the Hh signaling pathway through exosomes, two different isolation techniques were employed to obtain exosome samples (CE, exosomes isolated from medium harvested from cells) and blank samples (BE, medium subjected to the same exosome isolation procedure as the medium harvested from cells). The techniques were ultracentrifugation and a salting-out procedure using commercial isolation kit. Ultracentrifugation includes a series of centrifugation steps to remove cell debris with a subsequent high-speed ultracentrifugation to pellet the exosomes. The principle of the salting-out procedure is that water molecules are tied up by a reagent and forces less-soluble components (as exosomes) out of solution allowing them to be collected by lower speed centrifugation. The use of ultracentrifugation does not discriminate between exosomes and other vesicles or proteins [37] while with the salting-out procedure, other less soluble components can be isolated. Therefore, other methods should be employed in combination with the mentioned techniques to verify that exosomes have been isolated. Such methods can be e.g. Nanoparticle Tracking Analysis (where an instrument counts nanoparticles (as exosomes, 40-100 nm) in liquid suspensions), western blotting (WB) and/or proteomics. With the use of WB and/or proteomics, proteins specific for exosomes can be detected. Such conformational analysis has not been performed in this study.

## **3.3 Evaluation of method for determination of oxysterols in exosomes**

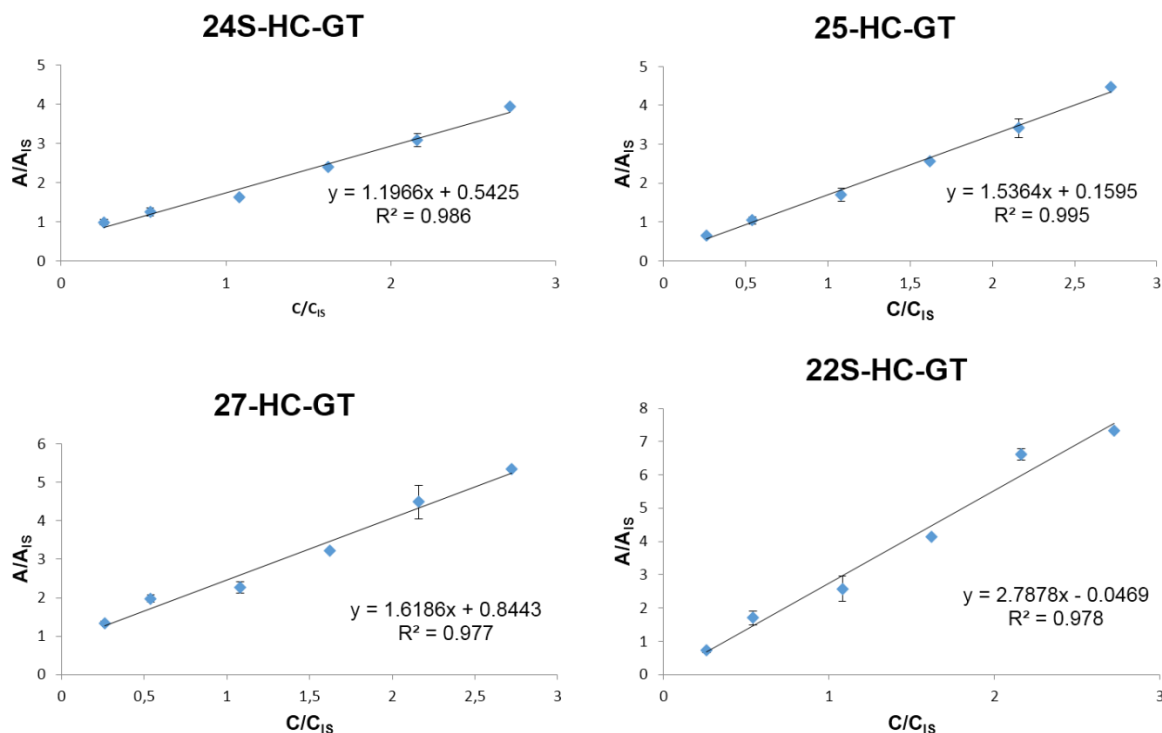
The modified method intended for determining Girard T derivatized oxysterols in exosomes has not been fully validated due to the time-restrictions of this study. However, parameters as selectivity, linearity, M and D and cholesterol autoxidation have been discussed and/or examined. Ideally, the standard solutions used to evaluate these parameters should have been based on the same matrix as present in samples. However, such matrix is not available as cells contain oxysterols; therefore, standard solutions without sample matrix and spiked cell lysate samples have been employed.

### 3.3.1 Selectivity

Selectivity is defined as how well the method can distinguish between an analyte and other components in a sample including other analytes, matrix component and other potential interferences. By employing MS/MS for detection, the method is selective for Girard T derivatives of oxysterols as only molecular ions with the same precursor ion mass and fragment ion mass as analytes are detected. Nevertheless, when determining oxysterol isomers from samples possible co-elution of unidentified isomers can occur. This can be examined by including other known oxysterol standards in the method used.

### 3.3.2 Linearity

Method linearity have not been examined, however, linearity was investigated in the range 14-136 pM of 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT. Good linearity was observed for 25-HC-GT ( $R^2 > 0.99$ ) while for 25S-HC-GT, 27-HC-GT and 22S-HC-GT  $R^2$  was somewhat lower ( $R^2 < 0.99$ ), as shown in Figure 24. A possible explanation may be that with the chromatographic conditions used, some peak overlapping occurs ( $R_s \sim 1-1.3$ ) that can make accurate integrations more difficult than with full resolution (i.e.  $R_s \geq 1.5$ ). This can also explain why the regression lines do not intersect origo.



**Figure 24: Linearity investigation for the concentrations 14-136 pM for 24S-HC-GT ( $R^2 < 0.99$ ), 25-HC-GT ( $R^2 > 0.99$ ), 27-HC-GT ( $R^2 < 0.99$ ) and 22S-HC-GT ( $R^2 < 0.99$ ). Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.**

### 3.3.3 Matrix effects

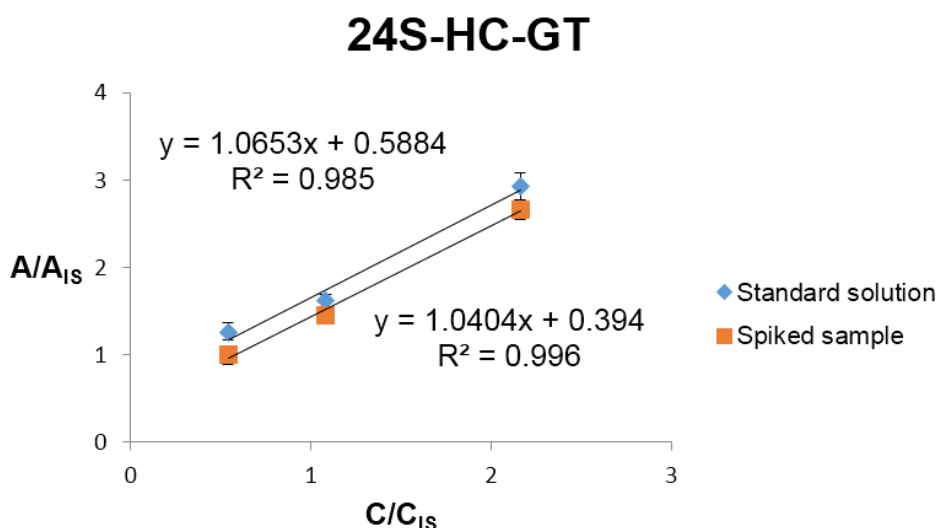
Matrix effects are a measure of the influence of interferences in the response of analytes due to the presence of unintended analytes or other interfering substances in the sample. This could have been examined by comparing standards and blank samples in MS mode. If there is positive or negative response from other components in MS at the same retention time as the analytes, then matrix effects are present giving either suppression or enhancement of the ion signal. If there is no response at the same retention time as the analytes, then no matrix effects have been addressed. As an analyte-free biological matrix is not available, an alternate analyte-free matrix could have been used to examine matrix effects, but due to time restrictions of this study, investigations of matrix effects have not been fully performed.

### 3.3.4 Completeness of the derivatization reaction and matrix effects

D and M can affect the ionization of analytes (oxysterols) during analysis. To assess this, analysis of both spiked cell samples and standard solutions at three concentration levels, (27 pM, 54 pM and 108 pM) were performed. Ideally, this should have been performed with the use of spiked CEs as well, but these samples were not available at the time of study. M and D were calculated as described in section 2.6.2 and it was calculated to be above 75% for all the oxysterols. The apparent recovery of M and D is presented in Table 4 and linear curves obtained from this experiment are shown in Figure 25-28.

**Table 4: Apparent recovery (M and D) for the derivatized oxysterols analytes.**

Analyte	24S-OHC-GT	25-OHC-GT	27-OHC-GT	22S-OHC-GT
Recovery (M and D) (%)	98	81	92	79



**Figure 25: Spiked cell lysate samples (n = 3 injections) and standard solutions (n = 3) used to calculate M and D of 24S-HC-GT. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.**

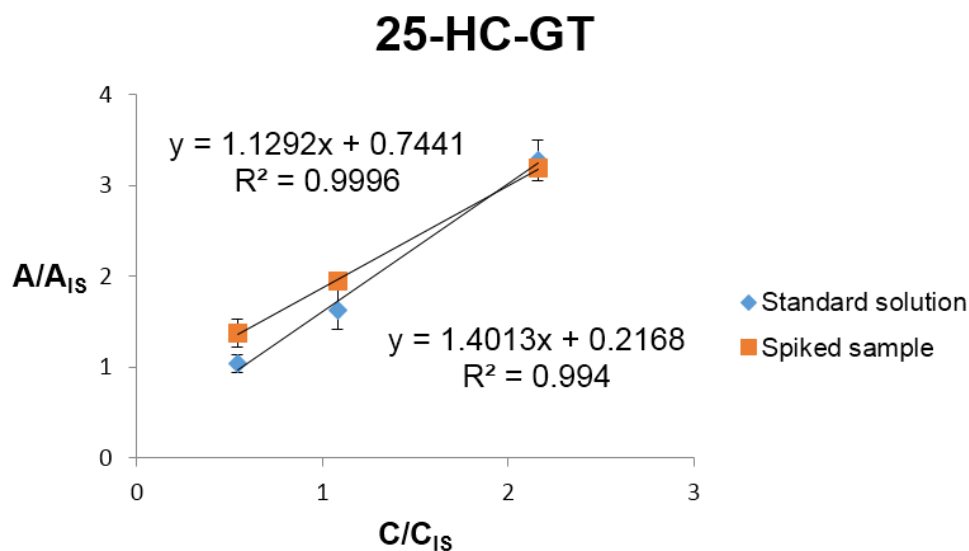


Figure 26: Spiked cell lysate samples (n = 3 injections) and standard solutions (n = 3) used to calculate M and D of 25-HC-GT. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.

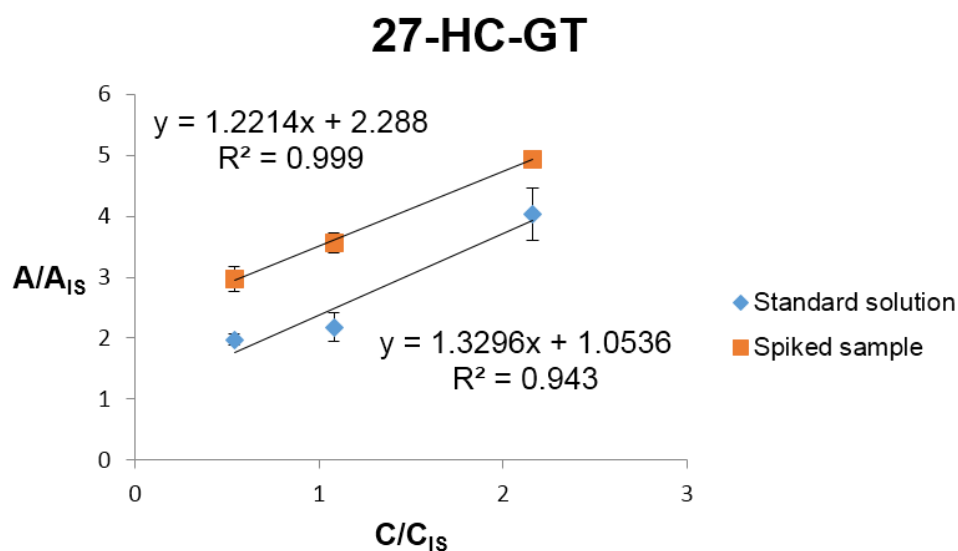
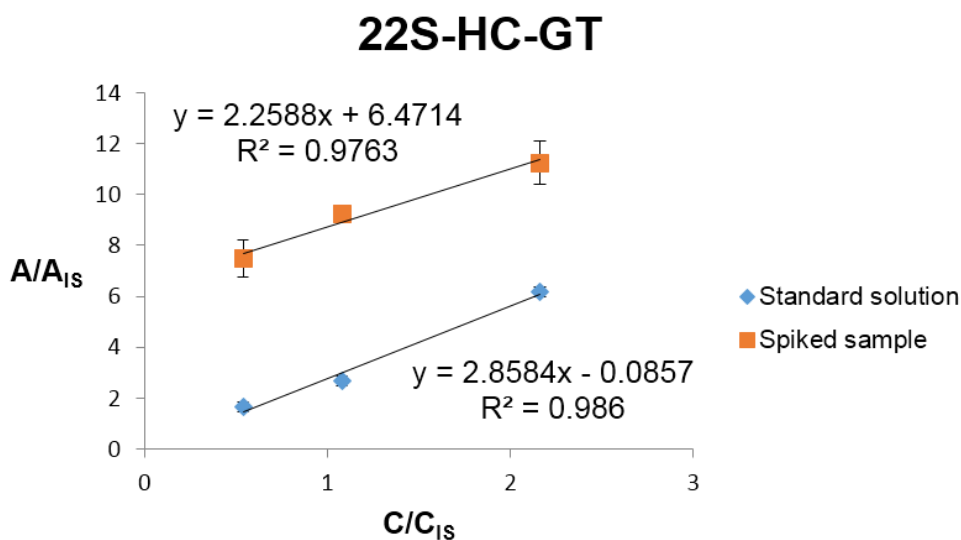


Figure 27: Spiked cell lysate samples (n = 3 injections) and standard solutions (n = 3) used to calculate M and D of 27-HC-GT. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.

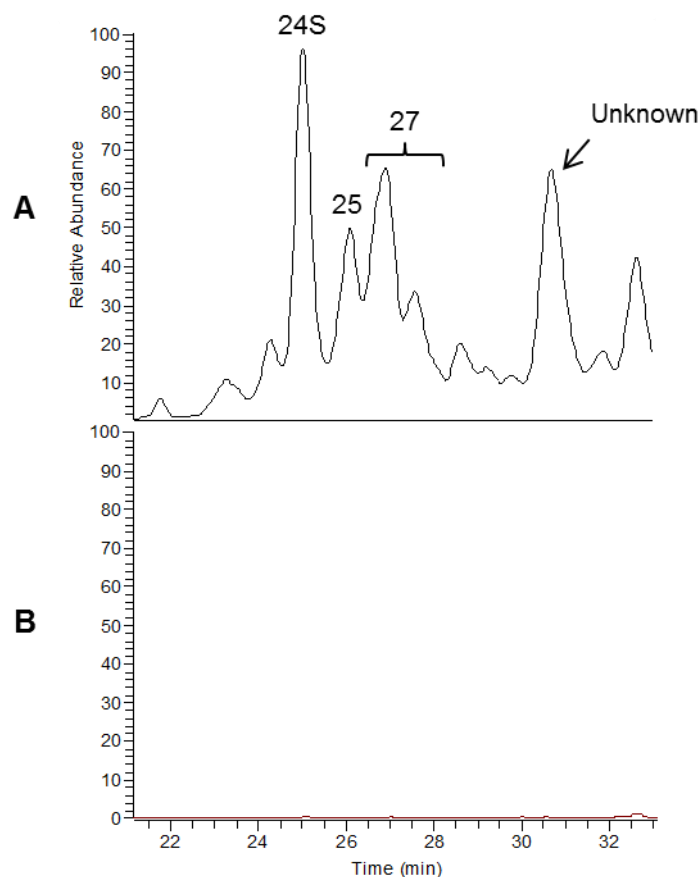


**Figure 28:** Spiked cell lysate samples (n = 3 injections) and standard solutions (n = 3) used to calculate M and D of 22S-HC-GT. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.

### 3.3.5 Cholesterol autoxidation

In samples autoxidation of cholesterol to oxysterols may occur if the sample is subjected to heat or light and this can give false positive results. By adding 25,26,27  $^{13}\text{C}$  cholesterol (100  $\mu\text{L}$  400 nM 25,26,27  $^{13}\text{C}$  cholesterol, 40 pmol) to the CE, autoxidation from sample preparation and storage conditions can be revealed by monitoring heavy oxysterols in MS/MS. A representative chromatogram in Figure 29 shows that the presence of autoxidation products (with the same retention time as the analytes) in exosome samples was negligible.



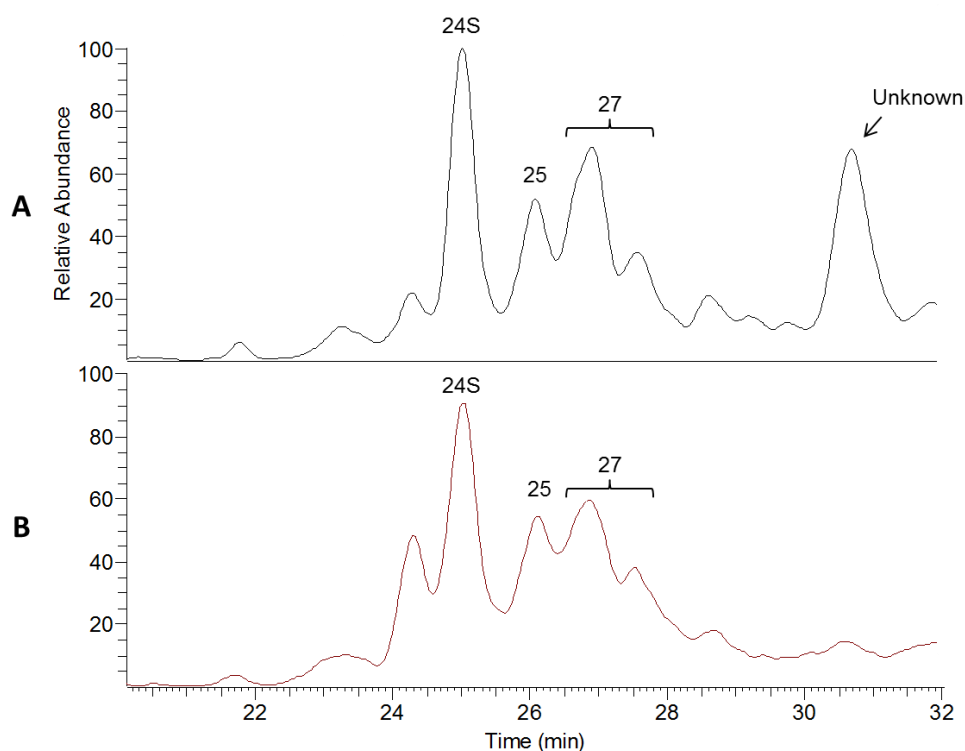


**Figure 29:** EIC of (A) derivatized oxysterols ( $m/z$  514.44  $\rightarrow$  455.36) and (B) autoxidation internal standard ( $m/z$  517.44  $\rightarrow$  458.36, bottom) from CE. No autoxidation products were found. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C. Signal intensities are normalized with regard to A.

### 3.4 Determination of oxysterols in exosomes

As mentioned in the introduction, it is of interest to examine the presence of oxysterols in exosome samples and some preliminary experiments, performed by the author of this thesis and Hanne Røberg-Larsen, are presented in the following. Determination of derivatized oxysterols 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT in CE and BE were performed with the same conditions as described in section 3.2.6. Representative chromatograms (EIC of  $m/z$  514.44  $\rightarrow$  455.36) in Figure 30 shows that 24S-HC-GT, 25-HC-GT, 27-HC-GT in addition to an unknown peak (most probably an unidentified oxysterol) are

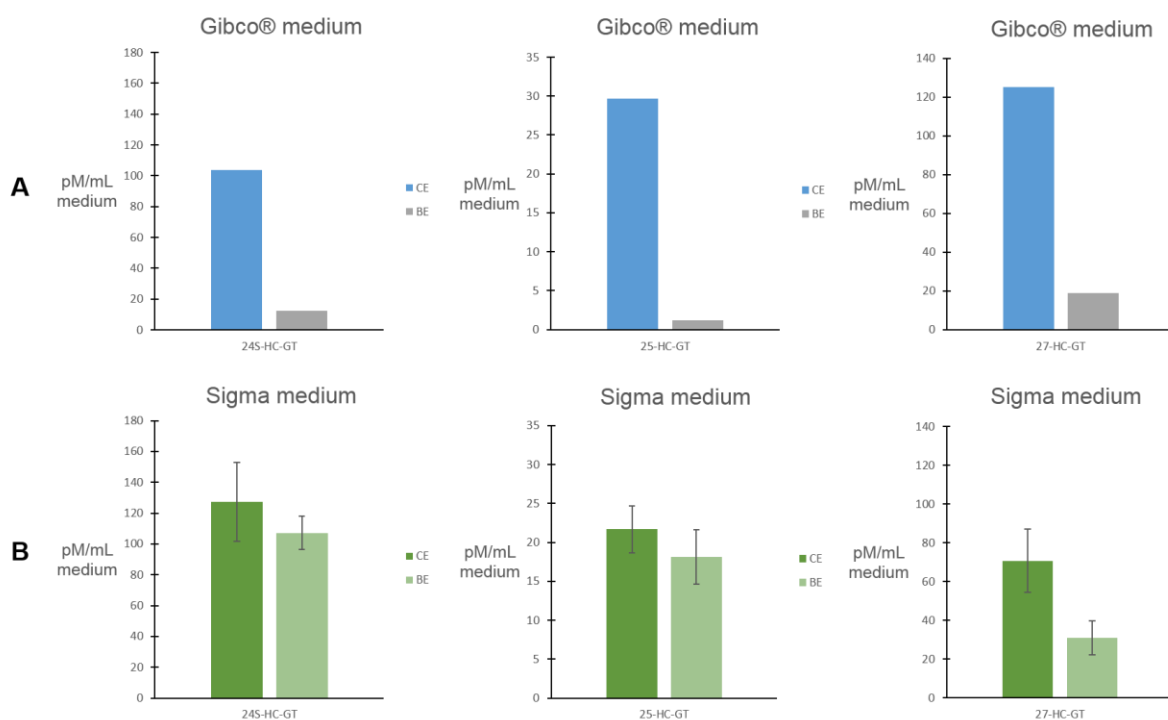
found in CE. Consistent with literature [65], 22S-HC-GT was not detected above the concentration limit of detection (cLOD ~14 pM) in these biological samples. The same oxysterol derivatives identified in CE were also identified in BE, except for the unknown peak.



**Figure 30: EIC of derivatized oxysterols ( $m/z$  514.44  $\rightarrow$  455.36) in (A) CE (B) BE. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C. Signal intensities are normalized with regard to A.**

The level of oxysterols in CE and BE with the use of different media types was also investigated. Ideally, the concentration of the oxysterol analytes in BE should be lower than the cLOD of the oxysterols. If not, false positives can affect the results. As seen from the bar graph in Figure 31, oxysterols were detected in both the Sigma and Gibco® medium. The level of oxysterols in BE was however lower in the Gibco® medium compared with the Sigma medium. Due to the high concentration of oxysterols in the Sigma medium, the medium cannot be used further. However, the Gibco® medium may be used in the future, if

(possible) exosomes are removed though e.g. ultracentrifugation before the medium is used to cultivate cells. Another option can be to examine the concentration of oxysterols in media from other vendors.



**Figure 31: Comparison of oxysterols concentration (pM/mL medium) of 24S-HC-GT, 25-HC-GT and 27-HC-GT from CE and BE with (A) Gibco® medium (n = 1 for CE and BE) and (B) Sigma medium (n = 6 for CE and n = 4 for BE), showing that BE obtained with Gibco® medium contains less exosomes and/or oxysterols compared with BE obtained with Sigma medium. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.**

### 3.4.1 Evaluation of harvest period, isolation technique and volume culture medium

From an earlier experiment, variations (RSD ~20-60%) in concentration (~10-30 pM) of the different oxysterols were observed for CE and BE harvested after 24 hours. A harvest period of 72 hours gave more repeatable concentrations (RSD 14-28%). This can be explained by a higher concentration of exosomes (and oxysterols) in samples harvested after 72 hours due to

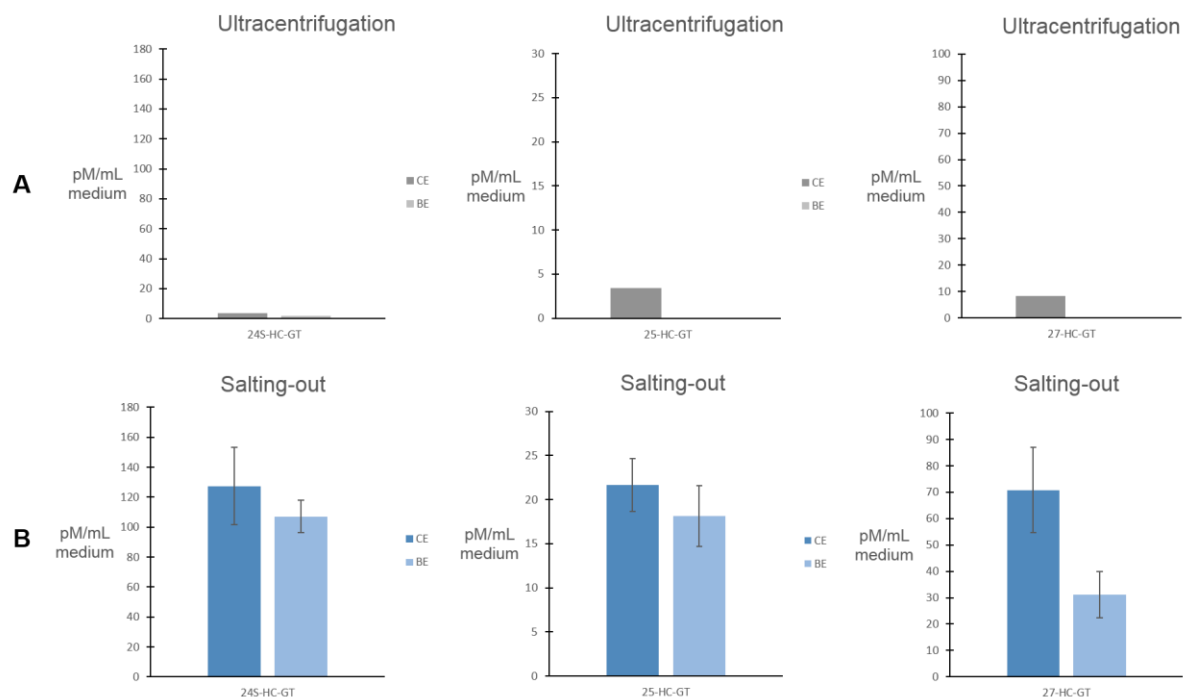
e.g. cell proliferation, which potentially can result in an increased level of oxysterols (and oxysterols) in the culture media. Therefore, a harvest period for 72 hours was used further.

The two different isolation techniques employed were compared. A description of the different samples is given in Table 5.

**Table 5: Description of CE and BE used to investigate concentration of oxysterols in exosomes.**

<b>CE</b>	<b>BE</b>	<b>Harvest period (hours)</b>	<b>Culture medium type (mL)</b>	<b>Isolation technique</b>
E72-1mL	B72-1mL	72	Sigma (1 mL)	Salting-out
E72-3mL	B72-3mL	72	Gibco® (3 mL)	Salting-out
ES	BS	72	Sigma (50 mL)	Ultracentrifugation

Quantification of analytes (oxysterols) in exosomes is difficult, as the number of possible exosomes were not counted. Instead, concentration in pM/mL culture medium was calculated to compare isolation techniques. The graphic presentation in Figure 32 shows that the concentrations of oxysterols from CE and BE obtained with ultracentrifugation were lower compared with the use of the salting-out procedure. A possible explanation may be that ultracentrifugation is a less effective isolation technique as discussed in section 3.2.7. Also, the procedure for ultracentrifugation included an extra washing step of the pellet formed, which might affect the yield of isolation.



**Figure 32: A comparison of the concentration of 24S-OHC-GT, 25-HC-GT and 27-HC-GT (pM/mL medium) in CE and BE, obtained by using the (A) ultracentrifugation technique (ES and BS, n = 1) and (B) the salting-out technique (E72-1mL (n = 6) and B72-1mL (n = 4)). The salting-out technique is possibly a more effective technique for isolation of exosomes (containing oxysterols) as a higher concentration of oxysterols are observed. Chromatographic conditions were as described in Figure 19 and separation was performed with gradient mobile phase elution from 75-90% B in 25 minutes with a column temperature of 15°C.**

The concentration of the oxysterols in CE is outside the range 14-136 pM in E72-3mL and ES while it is within the range in E72-1mL, as seen in Table 6. The concentration (pM) of 24-HC-GT, 25-HC-GT and 27-HC-GT in the different samples indicate that a sufficient volume of medium used to isolate exosomes from varies. As mentioned, the Sigma medium is not recommended to be used further due to contamination of exosomes and/or oxysterols. However, the use of Gibco® medium is more appropriate and less than 3 mL (conceivably 1 mL) can be a suitable harvest volume (after cultivation of cells).

**Table 6: Concentration (pM) of 24S-HC-GT, 25-HC-GT and 27-HC-GT in different CE samples (E72-1mL (n = 6), E72-3mL (n = 1) and ES (n = 1)).**

<b>CE (medium)</b>	<b>pM 24S-HC-GT (RSD %)</b>	<b>pM 25-HC-HT (RSD %)</b>	<b>pM 27-HC-GT (RSD %)</b>
<b>E72-1mL (Sigma)</b>	127 (20)	22 (14)	71 (23)
<b>E72-3mL (Gibco®)</b>	311	89	376
<b>ES (Gibco®)</b>	174	170	408

The concentrations of oxysterols found in CE and BE implies that the salting-out procedure is a more effective isolation technique and can be recommended for future experiments. In addition, the salting-out procedure is easier to use and can be more time effective. Cultivated cell medium can be harvested after 72 hours and the volume of culture medium (after cultivation of cells) can e.g. be ~1 mL for the Gibco® medium. As the Sigma medium shows a higher background of oxysterols, the Gibco® medium seems more applicable to use in future experiments. However, it can be recommended to investigate other media types with a possible lower concentration of exosomes (and/or oxysterols) or centrifuge the Gibco® medium and thereby pelleting and remove possible exosomes prior to use.

## 4 Conclusion

Sensitive determination of native oxysterols with the use of adduct formation to potentially reduce the time and complexity of the sample preparation steps, necessary in the established method, was not possible due to unstable ion signals with the MS instruments available. Relatively stable loss of water ions of native oxysterols was observed implying successful MS detection by this mode. However introduction of native oxysterols into the nanoLC system resulted in persistent carry-over. Several attempts to locate the origin of carry-over in addition to the fused silica adsorption effect were made, without success. Hence, sensitive determination of native oxysterols was considered unfeasible.

For the purpose to determine the low concentration of oxysterols in complex biological samples, derivatization with Girard T reagent can be more suitable approach. Reliable MS/MS detection is easily obtained and the AFFL-SPE-nanoLC system is free from carry-over issues. The modified method provided applicable chromatographic separation of the Girard T derivatized oxysterols and was successfully used to investigate the presence of oxysterols in exosomes. Different parameters as harvest period, isolation technique, volume of culture medium and medium type were evaluated to find a suitable approach to isolate exosomes from cell cultured medium.

To isolate exosomes, the salting-out technique seems more applicable compared with ultracentrifugation due to a higher concentration of oxysterols (and/or exosomes) and can be recommended to be used further. The Gibco® medium should be used rather than Sigma medium because of its lower oxysterol (and/or exosome) concentration however; medium from other vendors should preferably be investigated or the medium can possibly be ultracentrifuged prior to use. In addition, conformational analysis should be performed to validate isolation of exosomes from the cell culture medium.

## 5 References

1. Iuliano, L., *Pathways of cholesterol oxidation via non-enzymatic mechanisms*. Chemistry and Physics of Lipids, 2011. **164**(6): p. 457-468.
2. Brown, A.J. and W. Jessup, *Oxysterols: Sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis*. Molecular Aspects of Medicine, 2009. **30**(3): p. 111-122.
3. Nelson, E.R., S.E. Wardell, and D.P. McDonnell, *The molecular mechanisms underlying the pharmacological actions of estrogens, SERMs and oxysterols: Implications for the treatment and prevention of osteoporosis*. Bone, 2013. **53**(1): p. 42-50.
4. Nelson, E.R., et al., *The oxysterol, 27-hydroxycholesterol, links cholesterol metabolism to bone homeostasis through its actions on the estrogen and liver X receptors*. Endocrinology, 2011. **152**(12): p. 4691-4705.
5. Roberg-Larsen, H., et al., *Metabolites in vertebrate Hedgehog signaling*. Biochemical and Biophysical Research Communications, 2014. **446**(3): p. 669-674.
6. Pikuleva, I.A., *Cholesterol-metabolizing cytochromes P450: Implications for cholesterol lowering*. Expert Opinion on Drug Metabolism and Toxicology, 2008. **4**(11): p. 1403-1414.
7. Ohyama, Y., et al., *Studies on the transcriptional regulation of cholesterol 24-hydroxylase (CYP46A1): Marked insensitivity toward different regulatory axes*. Journal of Biological Chemistry, 2006. **281**(7): p. 3810-3820.
8. Lund, E.G., et al., *cDNA cloning of mouse and human cholesterol 25-hydroxylases, polytopic membrane proteins that synthesize a potent oxysterol regulator of lipid metabolism*. Journal of Biological Chemistry, 1998. **273**(51): p. 34316-34327.
9. Myers, B.R., et al., *Hedgehog pathway modulation by multiple lipid binding sites on the smoothed effector of signal response*. Developmental Cell, 2013. **26**(4): p. 346-357.
10. Nachtergaele, S., et al., *Oxysterols are allosteric activators of the oncoprotein Smoothed*. Nature Chemical Biology, 2012. **8**(2): p. 211-220.
11. Nedelcu, D., et al., *Oxysterol binding to the extracellular domain of Smoothed in Hedgehog signaling*. Nature Chemical Biology, 2013. **9**(9): p. 557-564.
12. Nachtergaele, S., et al., *Structure and function of the Smoothed extracellular domain in vertebrate Hedgehog signaling*. Elife, 2013. **2**: p. 1-32.
13. Ingham, P.W. and A.P. McMahon, *Hedgehog signaling in animal development: Paradigms and principles*. Genes and Development, 2001. **15**(23): p. 3059-3087.
14. Briscoe, J. and P.P. Therond, *The mechanisms of Hedgehog signalling and its roles in development and disease*. Nature Reviews Molecular Cell Biology, 2013. **14**(7): p. 416-429.
15. Teperino, R., et al., *Hedgehog Partial Agonism Drives Warburg-like Metabolism in Muscle and Brown Fat*. Cell, 2012. **151**(2): p. 414-426.
16. Harwell, C.C., et al., *Sonic hedgehog expression in corticofugal projection neurons directs cortical microcircuit formation*. Neuron, 2012. **73**(6): p. 1116-1126.
17. Babcock, D.T., et al., *Hedgehog signaling regulates nociceptive sensitization*. Current Biology, 2011. **21**(18): p. 1525-1533.
18. Amakye, D., Z. Jagani, and M. Dorsch, *Unraveling the therapeutic potential of the Hedgehog pathway in cancer*. Nature Medicine, 2013. **19**(11): p. 1410-1422.



19. Barakat, M.T., E.W. Humke, and M.P. Scott, *Learning from Jekyll to control Hyde: Hedgehog signaling in development and cancer*. Trends in Molecular Medicine, 2010. **16**(8): p. 337-348.
20. Cohen Jr, M.M., *Hedgehog signaling update*. American Journal of Medical Genetics, Part A, 2010. **152**(8): p. 1875-1914.
21. Johnson, R.L., et al., *Human homolog of patched, a candidate gene for the basal cell nevus syndrome*. Science, 1996. **272**(5268): p. 1668-1671.
22. Mullor, J.L., P. Sánchez, and A. Ruiz i Altaba, *Pathways and consequences: Hedgehog signaling in human disease*. Trends in Cell Biology, 2002. **12**(12): p. 562-569.
23. Teglund, S. and R. Toftgård, *Hedgehog beyond medulloblastoma and basal cell carcinoma*. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2010. **1805**(2): p. 181-208.
24. Yang, L., et al., *Activation of the hedgehog-signaling pathway in human cancer and the clinical implications*. Oncogene, 2010. **29**(4): p. 469-481.
25. Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila*. Nature, 1980. **287**(5785): p. 795-801.
26. Van den Heuvel, M. and P.W. Ingham, *Smoothed encodes a receptor-like serpentine protein required for hedgehog signalling*. Nature, 1996. **382**(6591): p. 547-551.
27. Alcedo, J., et al., *The Drosophila smoothed gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal*. Cell, 1996. **86**(2): p. 221-232.
28. Tiniakow, G.G. and E.L. Terentieva, *Cubitus Interruptus, a New Genovariation of the Fourth Chromosome of DROSOPHILA MELANOGASTER*. Genetics, 1933. **18**(2): p. 117-120.
29. Orenic, T.V., et al., *Cloning and characterization of the segment polarity gene cubitus interruptus Dominant of Drosophila*. Genes and Development, 1990. **4**(6): p. 1053-1067.
30. Marigo, V., et al., *Biochemical evidence that Patched is the Hedgehog receptor*. Nature, 1996. **384**(6605): p. 176-179.
31. Echelard, Y., et al., *Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity*. Cell, 1993. **75**(7): p. 1417-1430.
32. Akiyama, H., et al., *Cloning of a mouse smoothed cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5*. Biochemical and Biophysical Research Communications, 1997. **235**(1): p. 142-147.
33. Rana, R., et al., *Structural insights into the role of the Smoothed cysteine-rich domain in Hedgehog signalling*. Nature Communications, 2013. **4**: p. 1-9.
34. Chen, J.K., et al., *Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothed*. Genes and Development, 2002. **16**(21): p. 2743-2748.
35. Wang, C., et al., *Structure of the human smoothed receptor bound to an antitumour agent*. Nature, 2013. **497**(7449): p. 338-343.
36. Aanstad, P., et al., *The Extracellular Domain of Smoothed Regulates Ciliary Localization and Is Required for High-Level Hh Signaling*. Current Biology, 2009. **19**(12): p. 1034-1039.
37. Théry, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function*. Nature Reviews Immunology, 2002. **2**(8): p. 569-579.
38. Février, B. and G. Raposo, *Exosomes: endosomal-derived vesicles shipping extracellular messages*. Current Opinion in Cell Biology, 2004. **16**(4): p. 415-421.

39. Simons, M. and G. Raposo, *Exosomes—vesicular carriers for intercellular communication*. Current Opinion in Cell Biology, 2009. **21**(4): p. 575-581.
40. Raposo, G. and W. Stoorvogel, *Extracellular vesicles: exosomes, microvesicles, and friends*. The Journal of Cell Biology, 2013. **200**(4): p. 373-383.
41. Mignot, G., et al., *Prospects for exosomes in immunotherapy of cancer*. Journal of Cellular and Molecular Medicine, 2006. **10**(2): p. 376-388.
42. Lakkaraju, A. and E. Rodriguez-Boulan, *Itinerant exosomes: emerging roles in cell and tissue polarity*. Trends in Cell Biology, 2008. **18**(5): p. 199-209.
43. Zhou, H., et al., *Urinary exosomal transcription factors, a new class of biomarkers for renal disease*. Kidney International, 2008. **74**(5): p. 613-621.
44. Pisitkun, T., R. Johnstone, and M.A. Knepper, *Discovery of urinary biomarkers*. Molecular & Cellular Proteomics, 2006. **5**(10): p. 1760-1771.
45. Bischoff, M., et al., *Cytogenes are required for the establishment of a normal Hedgehog morphogen gradient in Drosophila epithelia*. Nature Cell Biology, 2013. **15**(11): p. 1269-1281.
46. Vyas, N., et al., *Vertebrate Hedgehog is secreted on two types of extracellular vesicles with different signaling properties*. Scientific Reports, 2014. **4**(7357): p. 2045-2322.
47. Riches, A., et al., *Regulation of exosome release from mammary epithelial and breast cancer cells—a new regulatory pathway*. European Journal of Cancer, 2014. **50**(5): p. 1025-1034.
48. Griffiths, W.J., et al., *Discovering oxysterols in plasma: a window on the metabolome*. Journal of Proteome Research, 2008. **7**(8): p. 3602-3612.
49. Wang, Y. and W.J. Griffiths, *Capillary liquid chromatography combined with tandem mass spectrometry for the study of neurosteroids and oxysterols in brain*. Neurochemistry International, 2008. **52**(4-5): p. 506-521.
50. Ogden, P.B. and J.W. Coym, *Retention mechanism of a cholesterol-coated C18 stationary phase: van't Hoff and Linear Solvation Energy Relationships (LSER) approaches*. Journal of Chromatography A, 2011. **1218**(20): p. 2936-2943.
51. Axelson, M., B. Mörk, and J. Sjövall, *Occurrence of 3 beta-hydroxy-5-cholestenoic acid, 3 beta, 7 alpha-dihydroxy-5-cholestenoic acid, and 7 alpha-hydroxy-3-oxo-4-cholestenoic acid as normal constituents in human blood*. Journal of Lipid Research, 1988. **29**(5): p. 629-641.
52. Axelson, M. and J. Sjövall, *Potential bile acid precursors in plasma—possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man*. Journal of Steroid Biochemistry, 1990. **36**(6): p. 631-640.
53. Axelson, M., B. Mörk, and G. Everson, *Bile acid synthesis in cultured human hepatoblastoma cells*. Journal of Biological Chemistry, 1991. **266**(27): p. 17770-17777.
54. Dzeletovic, S., et al., *Determination of Cholesterol Oxidation Products in Human Plasma by Isotope Dilution-Mass Spectrometry*. Analytical Biochemistry, 1995. **225**(1): p. 73-80.
55. Karu, K., et al., *Nano-liquid chromatography–tandem mass spectrometry analysis of oxysterols in brain: monitoring of cholesterol autoxidation*. Chemistry and Physics of Lipids, 2011. **164**(6): p. 411-424.
56. Griffiths, W.J. and Y. Wang, *Analysis of oxysterol metabolomes*. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2011. **1811**(11): p. 784-799.
57. Honda, A., et al., *Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC–ESI–MS/MS*. The Journal of Steroid Biochemistry and Molecular Biology, 2010. **121**(3): p. 556-564.

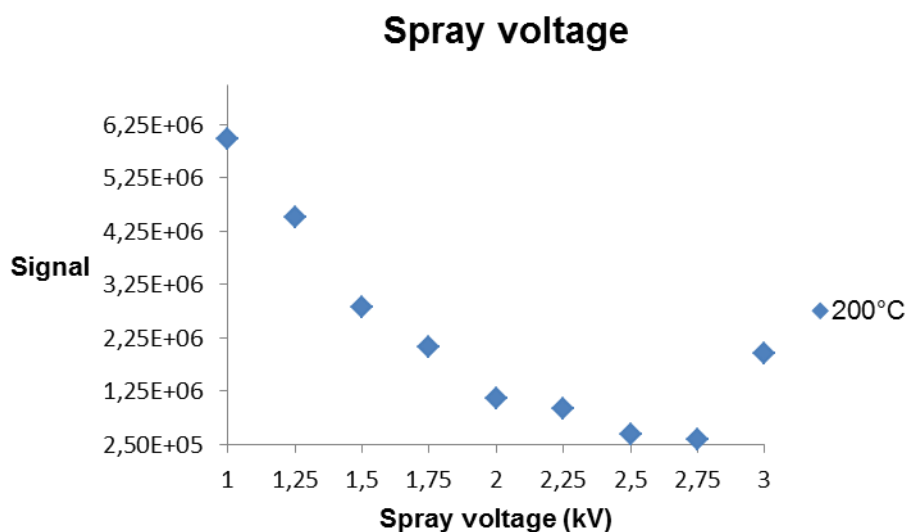
58. Ahonen, L., et al., *Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure photoionization–mass spectrometry*. Journal of Chromatography A, 2014. **1364**: p. 214-222.
59. Roberg-Larsen, H., et al., *High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography–mass spectrometry*. Journal of Chromatography A, 2012. **1255**: p. 291-297.
60. Limited, A.C.T. ACE® C18-PFP A C18 bonded phase with unique selectivity. [cited 2015 29/04/15]; Available from: <http://www.ace-hplc.com/products/product.aspx?id=116>.
61. DeBarber, A.E., et al., *Liquid chromatography–tandem mass spectrometry determination of plasma 24S-hydroxycholesterol with chromatographic separation of 25-hydroxycholesterol*. Analytical Biochemistry, 2008. **381**(1): p. 151-153.
62. Burkard, I., K.M. Rentsch, and A. von Eckardstein, *Determination of 24S-and 27-hydroxycholesterol in plasma by high-performance liquid chromatography-mass spectrometry*. Journal of Lipid Research, 2004. **45**(4): p. 776-781.
63. McDonald, J.G., et al., *A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma*. Journal of Lipid Research, 2012. **53**(7): p. 1399-1409.
64. McDonald, J.G., et al., *Extraction and Analysis of Sterols in Biological Matrices by High Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry*, in *Methods in Enzymology*, H.A. Brown, Editor. 2007, Academic Press. p. 145-170.
65. Roberg-Larsen, H., et al., *Highly automated nanoLC-MS-based approach for thousand cell scale quantification of side chain-hydroxylated oxysterols*. Journal of Lipid Research, 2014(55): p. 1531-1536.
66. DeBarber, A.E., et al., *ESI-MS/MS quantification of 7 $\alpha$ -hydroxy-4-cholesten-3-one facilitates rapid, convenient diagnostic testing for cerebrotendinous xanthomatosis*. Clinica Chimica Acta, 2010. **411**(1): p. 43-48.
67. Griffiths, W.J., et al., *Analysis of Oxysterols by Electrospray Tandem Mass Spectrometry*. Journal of the American Society for Mass Spectrometry, 2006. **17**(3): p. 341-362.
68. Honda, A., et al., *Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS*. Journal of Lipid Research, 2009. **50**(2): p. 350-357.
69. Honda, A., et al., *Highly sensitive quantification of 7 $\alpha$ -hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS*. Journal of Lipid Research, 2007. **48**(2): p. 458-464.
70. Jiang, X., D.S. Ory, and X. Han, *Characterization of oxysterols by electrospray ionization tandem mass spectrometry after one-step derivatization with dimethylglycine*. Rapid Communications in Mass Spectrometry, 2007. **21**(2): p. 141-152.
71. Karu, K., et al., *Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols*. Journal of Lipid Research, 2007. **48**(4): p. 976-987.
72. Shackleton, C.H.L., et al., *Electrospray mass spectrometry of testosterone esters: Potential for use in doping control*. Steroids, 1997. **62**(7): p. 523-529.
73. Svendsen, K.O., et al., *Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography*. Journal of Separation Science, 2011. **34**(21): p. 3020-3022.

74. Kebarle, P. and U.H. Verkerk, *Electrospray: from ions in solution to ions in the gas phase, what we know now*. Mass Spectrometry Reviews, 2009. **28**(6): p. 898-917.
75. Banerjee, S. and S. Mazumdar, *Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte*. International Journal of Analytical Chemistry, 2012. **2012**: p. 1-40.
76. Waters. *Common Ionization*. 2015 [cited 2015 07/05/15]; Available from: [http://www.waters.com/waters/en\\_US/Common-Ionization/nav.htm?cid=10073251](http://www.waters.com/waters/en_US/Common-Ionization/nav.htm?cid=10073251).
77. Hardman, M. and A.A. Makarov, *Interfacing the orbitrap mass analyzer to an electrospray ion source*. Analytical Chemistry, 2003. **75**(7): p. 1699-1705.
78. Scientific, T. *Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometry*. 2015 [cited 2015 07/05/15]; Available from: <http://planetorbitrap.com/q-exactive#tab:schematic>.
79. Technologies™, I.b.L. *Total Exosome Isolation (from cell culture media)*. [cited 2015 06/05/15]; Available from: <http://www.lifetechnologies.com/order/catalog/product/4478359?CID=search-product>.
80. Rani, S., et al., *Isolation of exosomes for subsequent mRNA, MicroRNA, and protein profiling*, in *Gene Expression Profiling*. 2011, Springer. p. 181-195.
81. Seed, B., *Silanizing Glassware*, in *Current Protocols in Immunology*. 2001, John Wiley & Sons, Inc.
82. Røberg-Larsen, H., et al., *Underivatized oxysterols and nanoLC–ESI-MS: A mismatch*. Steroids, 2015(In Press).
83. Baila-Rueda, L., et al., *Simultaneous determination of oxysterols, phytosterols and cholesterol precursors by high performance liquid chromatography tandem mass spectrometry in human serum*. Analytical Methods, 2013. **5**(9): p. 2249-2257.
84. Røberg-Larsen, H., *Determination of oxysterols in cancer stem cells using on-line automated filtration and filter-flush solid phase extraction liquid chromatography tandem mass spectrometry*, in *Department of Chemistry*. 2011, University of Oslo. p. 99.

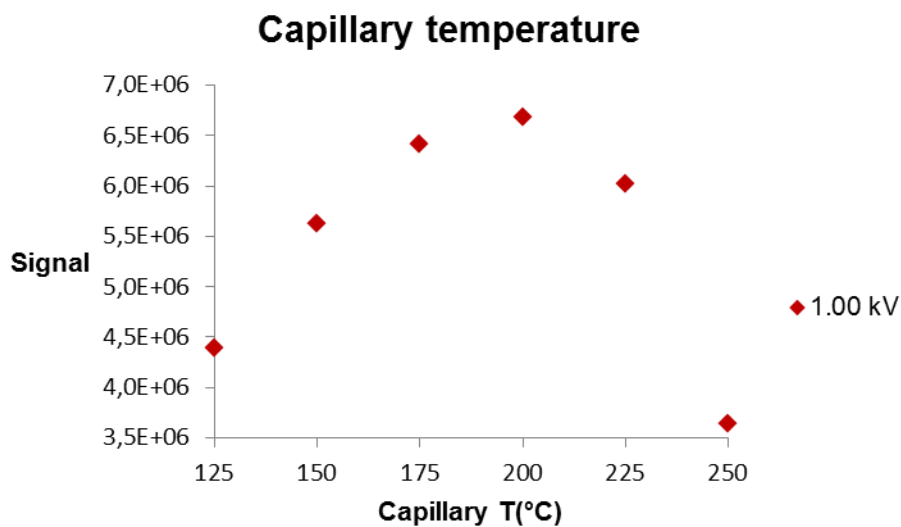
## 6 Appendix I

### 6.1 Signal intensity of acetate adducts of oxysterols

To achieve stable adduct ions between oxysterols and acetate, varying the spray voltage and the capillary temperature was investigated. An oxysterol standard solution (10 ng/ $\mu$ L 22S-OHC) was infused directly to the Q-Exactive™ Orbitrap MS instrument equipped with the Nanospray flex ESI source. First, the spray voltage was regulated stepwise from 1 kV to 3 kV while the capillary temperature was set to 200°C. The highest signal intensity of the adduct ions was obtained with a spray voltage of 1 kV (Figure 33). Subsequently, the capillary temperature was varied from 125°C to 250°C while maintaining a spray voltage of 1 kV. The uppermost signal intensity of the adduct ions was achieved with the combination of 1.00 kV and 200°C, see Figure 34.



**Figure 33:** Signal of adducts between oxysterols and acetate with a capillary temperature of 200°C and varying spray voltage from 1 kV to 3 kV. The highest signal intensity was obtained with a spray voltage of 1 kV.



**Figure 34:** Signal of adducts between oxysterols and acetate with a spray voltage from 1 kV and varying capillary temperature from 125-250<sup>0</sup>C. The highest signal intensity was obtained with a capillary temperature of 200<sup>0</sup>C.

## 6.2 Carry-over investigations

Various efforts to address and eliminate carry-over effects in the AFFL-SPE-nanoLC system, presented in Table 7, were made by the author of this thesis and Hanne Røberg-Larsen, without positive results.

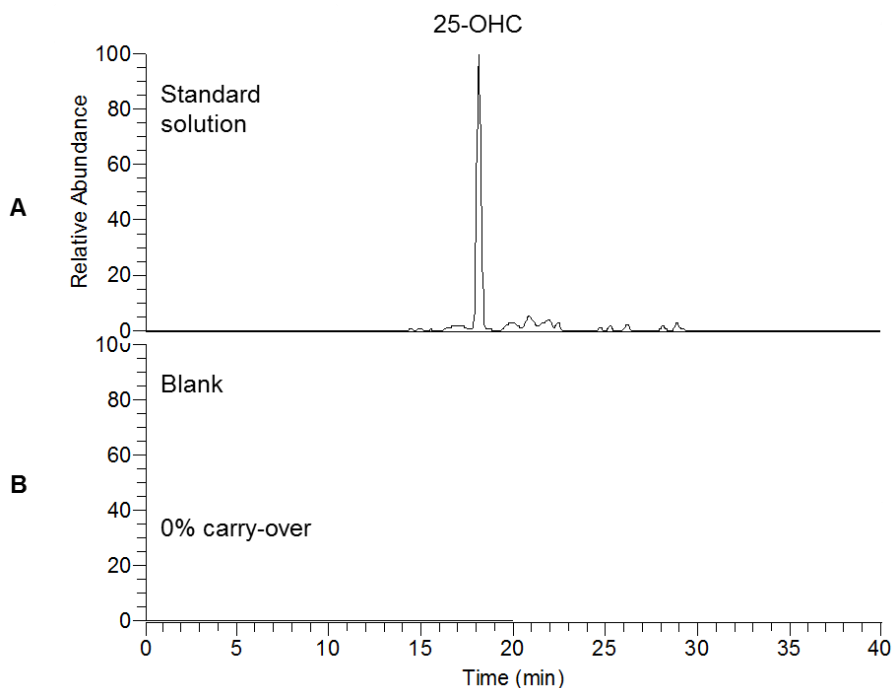
**Table 7: An overview of the efforts made to locate and eliminate carry-over in the AFFL-SPE-nanoLC system. Adapted from: [82].**

<b>Actions</b>	<b>Success?</b>	<b>Comments</b>
Changing all fused silica with silanized fused silica	No	Large carry -over after each injection, however carry-over is removed after approximately 60 minutes of washing with AF/FA in IPA.
Changing spray needle from steel to fused silica	No	
Injection with and without injection wash	No	
Flushing the injector system with strong solvent between analysis	No	
Switching injector rotor from bypass to main pass several times	No	
New blank sample for each injection	No	
Blank sample of other solvents	No	Both blank with higher solubility and lower solubility than mobile phase examined.
Changing SPE column from Kromasil material to ACE material	No	In house packed SPE with ACE material.
Changing SPE column to ACE material packed in silanized fused silica	No	In housed silanized and packed SPE with ACE material.
Changing SPE column to less hydrophobic material (from C <sub>18</sub> to C <sub>8</sub> and C <sub>4</sub> )	No	
Lower concentrations of injected solutions	No	10x diluted solutions, no MS signal.
Exchange rotor in 10 port valve and injector valve	No	
Exchange 10 port valve (completely)	No	
Manual injections with SPE	No	
Manual injections without SPE	No	
Exchange pump system	No	A Proxeon Easy nLC pump with two columns set up was tested. Carry-over observed after one injection (50%).

### 6.2.1 Carry-over in microLC

Investigation of carry-over effects was performed by injection of 800 pg/μL 25-OHC in a microLC system using a gradient mobile phase (MP A: 2.5 mM AF in H<sub>2</sub>O with 0.25% FA and MP B: 2.5 mM AF in MeOH with 0.25% FA) elution from 70-100% B in 10 minutes. In

this set-up a 1 mm ID C<sub>18</sub>-PFP column were employed connected to 100 µm ID fused silica tubing. As seen in Figure 35, carry-over was not observed.

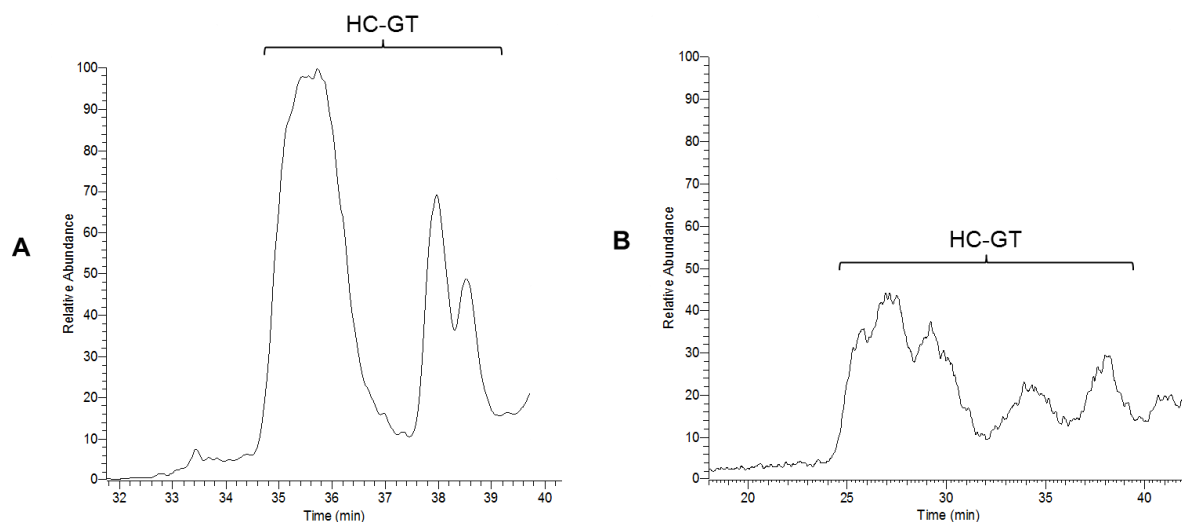


**Figure 35:** EIC ( $m/z$  367.34) of (A) 800 pg/mL 25-OHC and (B) MeOH, showing no carry-over in the microLC system. Analysis were performed on an ACE C<sub>18</sub>-PFP column (1mm ID × 150 mm) with gradient mobile phase (MP A: 2.5 mM AF in H<sub>2</sub>O with 0.25% FA and MP B: 2.5 mM AF in MeOH with 0.25% FA) 70-100% B in 10 minutes at 40 µL/min. Injection volume was 5 µL. Signal intensities are normalized with regard to A.

### 6.3 Separation with C<sub>18</sub>-PFP column functionality

Separation of derivatized oxysterol isomers with a C<sub>18</sub>-PFP column was performed with either gradient or isocratic mobile phase elution to improve the resolution obtained with the use of a C<sub>18</sub> column. A high degree of co-elution of the analytes was observed with the use of the C<sub>18</sub>-PFP column functionality (Figure 36). Therefore, a C<sub>18</sub> column functionality was used in the proceeding experiments.





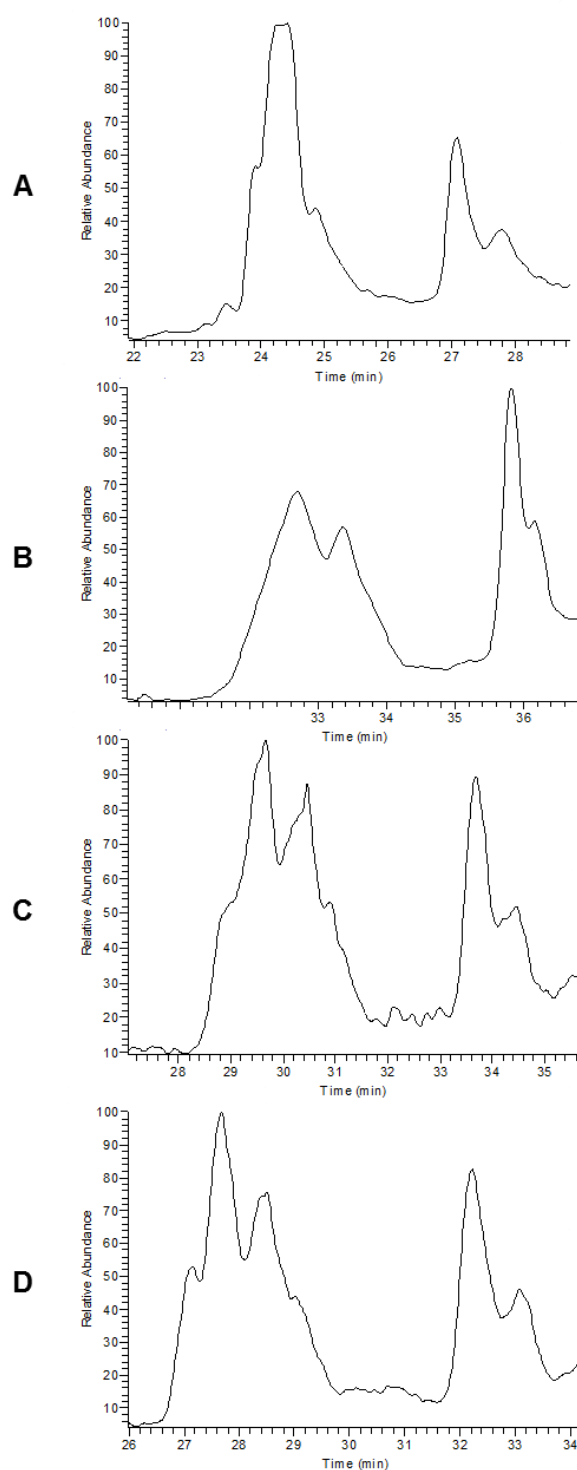
**Figure 36: Attempts for separating derivatized oxysterols (EIC (m/z 514.44 → 455.36)) by using a C<sub>18</sub>-PFP column (0.1 mm ID × 150 mm) with (A) gradient mobile phase elution, 75-90% B in 20 minutes and (B) isocratic elution 80% B. Other chromatographic conditions were as described in Figure 19. Signals are normalized with regard to A.**

## 6.4 Increased column length for improved column efficiency

An option to increase column efficiency is to use a longer column, as plate number (N) increases with increased column length (L), formula below.

$$N = \frac{L}{H}$$

Nonetheless, longer columns were not available; therefore, the combination of two ACE C<sub>18</sub> columns was investigated with the use of the AFFL-SPE-nanoLC system. This set-up was prone to high backpressure and a column temperature of 40°C was necessary to reduce backpressure to a pressure suitable for the pump. With the use of two columns, a high degree of co-elution of the derivatized oxysterol analytes was observed (Figure 37). The use of two columns to increase column efficiency was not successful and therefore not pursued.



**Figure 37: EIC (m/z 514.44 → 455.36) of 81 pM, 24S-HC-GT, 25-HC-GT, 27-HC-GT and 22S-HC-GT showing poor separation obtained from analysis using two ACE 3 C<sub>18</sub> columns coupled together with a column temperature of 40°C. Analysis was performed with the use of the AFFL-SPE-nanoLC system with either isocratic or gradient mobile phase elution (MP A: 0.1% FA in H<sub>2</sub>O and MP B: 0.1% FA in MeOH). (A) 95% B, (B) 60-90% B in 30 minutes, (C) 70-90% B in 25 minutes and (D) 75-90% B in 25 minutes. The injection volume was set to 5 µL.**

## 6.5 Raw data from exosome analysis

Raw data obtained from determination of oxysterols derivatives in CE and BE are shown in Table 8-10.

**Table 8: pM/mL medium 24S-HC-GT, 25-HC-GT and 27-HC-GT obtained from CE (E72-1mL, n = 6) with average, standard derivation (STD) and relative standard deviation (RSD %).**

<b>CE</b> <b>(n = 6)</b>	<b>24S-HC-GT</b> <b>pM/mL medium</b>	<b>25-HC-GT</b> <b>pM/mL medium</b>	<b>27-HC-GT</b> <b>pM/mL medium</b>
<b>E72-1mL#1</b>	83	24	70
<b>E72-1mL#2</b>	154	23	73
<b>E72-1mL#3</b>	114	23	78
<b>E72-1mL#4</b>	129	24	94
<b>E72-1mL#5</b>	137	18	45
<b>E72-1mL#6</b>	147	17	65
<b>Average</b>	127	22	71
<b>STD</b>	26	3	16
<b>RSD (%)</b>	20	14	23

**Table 9: pM/mL medium 24S-HC-GT, 25-HC-GT and 27-HC-GT obtained from BE (B72-1mL, n = 4) with average, STD and RSD (%).**

<b>BE</b> <b>(n = 4)</b>	<b>24S-HC-GT</b> <b>pM/mL medium</b>	<b>25-HC-GT</b> <b>pM/mL medium</b>	<b>27-HC-GT</b> <b>pM/mL medium</b>
<b>B72-1mL#1</b>	103	13	31
<b>B72-1mL#2</b>	97	19	42
<b>B72-1mL#3</b>	123	21	20
<b>B72-1mL#4</b>	105	19	32
<b>Average</b>	107	18	31
<b>SD</b>	11	4	9
<b>RSD (%)</b>	10	19	28

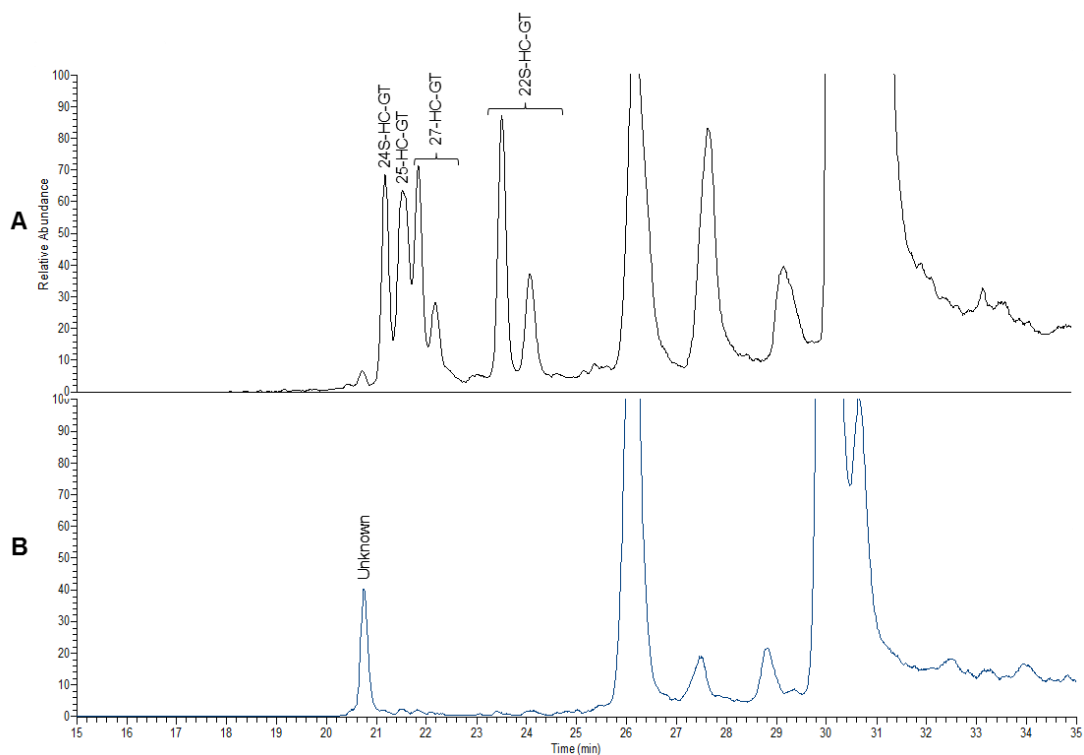
**Table 10: pM/mL medium 24S-HC-GT, 25-HC-GT and 27-HC-GT obtained from CE (E72-3mL, B72-3mL, ES and BS, n = 1).**

<b>Sample (n=1)</b>	<b>24S-HC-GT</b>	<b>25-HC-GT</b>	<b>27-HC-GT</b>
	<b>pM/mL medium</b>	<b>pM/mL medium</b>	<b>pM/mL medium</b>
<b>E72-3mL</b>	104	30	125
<b>B72-3mL</b>	12	1	19
<b>ES</b>	3	3	8
<b>BS</b>	2	0	0

## 6.6 Investigation of preexisting keto groups

The Girard T reagent reacts with keto group as shown in Figure 4 in section 1.4.3, without differentiating between oxysterols with keto groups formed after reaction with cholesterol oxidase and oxysterols with natural preexisting keto groups. To distinguish between natural keto groups and those formed during sample preparation, cell lysate samples were treated with and without cholesterol oxidase prior to charge tagging with Girard T reagent as described in [65]. This approach has the potential to discover false positive results if analytes are detected in the sample not treated with cholesterol oxidase.

Both samples were analyzed and no compounds eluted at the same time as 24S-OHC, 25-OHC, 27-OHC and 22S-HC-GT (Figure 38). This implies that determination of Girard T tagged oxysterols in the BxPC-3 cell lysate samples is safe as Girard T tagging of preexisting hydroxyl-4-cholesten-3-one was not detected. This should probably be tested if other biological samples obtained from different cell lines is analyzed with the aim to detect oxysterols charge tagged with a reagent specific to keto groups. Due to time restrictions of this study, this was not performed with regard to exosome samples.



**Figure 38: EIC ( $m/z$  514.44  $\rightarrow$  455.36) of cell lysate sample treated (A) with cholesterol oxidase and (B) without cholesterol oxidase. The chromatograms are aligned with the internal standard retention time and the signals are normalized. No detectable amount of 24S-HC-GT, 25-HC-GT, 27-HC-GT or 22S-HC-GT was found. Chromatographic conditions were as described in [65].**

# 7 Appendix II

## 7.1 Underivatized oxysterols and nanoLC-ESI-MS: A mismatch [82]



### Underivatized oxysterols and nanoLC-ESI-MS: A mismatch

Hanne Roberg-Larsen\*, Caroline Vesterdal, Steven Ray Wilson, Elsa Lundanes

Department of Chemistry, University of Oslo, PO Box 1033 Blindern, NO-0315 Oslo, Norway

#### ARTICLE INFO

**Article history:**  
Received 16 December 2014  
Received in revised form 13 January 2015  
Accepted 22 January 2015  
Available online xxx

**Keywords:**  
NanoLC  
Oxysterol  
Derivatization  
Mass spectrometry  
Girard reagent  
Carry-over

#### ABSTRACT

Due to their non-charged character, liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) measurements of oxysterols are often performed after derivatization with e.g. charged Girard reagents. However, derivatization reactions are time-consuming and may require numerous steps to remove excess reagent. In addition, extensive sample handling can be associated with cholesterol autoxidation, resulting in analyte artifacts and hence false positives.

Nano scale liquid chromatography in combination with electrospray–mass spectrometry (nanoLC–ESI–MS) is a powerful tool for analyzing limited samples, due to substantially increased sensitivity compared to conventional LC–ESI–MS. The signal enhancement may compensate for the poor ionization of the oxysterols; hence we have explored the possibility to quantify oxysterols without derivatization using nanoLC–ESI–MS.

Non-derivatized oxysterols and nanoLC were however not compatible, due to persistent and large carry-over. This was attributed to the extended contribution of surface to volume ratio in such miniaturized systems and interactions with the materials of the nanoLC instrumentation (e.g. adsorption to the fused silica tubing).

Two contemporary MS instruments (Q-Exactive™ hybrid quadrupole–Orbitrap and TSQ Quantiva™ triple quadrupole) were used. However, both the MS and MS/MS spectra of non-derivatized oxysterols were ambiguous and/or unrepeatable for both of the instruments employed.

Derivatizing oxysterols is more cumbersome, but provides more selective and reliable results, and Girard derivatization + nanoLC–ESI–MS continues to be our recommended choice for measuring oxysterols in very limited samples.

These investigations also indicate that extra care should be taken to remove lipids prior to nanoLC of other analytes, as adsorbed oxysterols, etc. can compromise analysis.

© 2015 Elsevier Inc. All rights reserved.

#### 1. Introduction

Oxysterols are hydroxylated cholesterol, formed either enzymatically or by autoxidation. Oxysterols have a great number of biological roles, dealing with e.g. regulation of cholesterol homeostasis [1], acting as selective estrogen receptor modulators [2], activating liver X receptors [3] and possibly the hedgehog signaling

pathway [4], in addition to being a biomarker for Niemann–Pick type C1 [5]. The neutral nature of oxysterols such as 24S-, 25- and 27-hydroxycholesterol (24S-OHC, 25-OHC and 27-OHC) makes their measurements challenging with ESI–MS, the standard instrumentation for identifying and measuring compounds separated by LC. Consequentially, several strategies for derivatizing oxysterols into chargeable species have been developed, e.g. derivatization with Girard reagents [6]. Such approaches can enable mass detection limits in pg–fg levels [6–10].

NanoLC (LC columns with inner diameters (ID) less than 0.1 mm) is a tool for increasing sensitivity in combination with ESI–MS (or other concentration-sensitive detectors), as compounds undergo significantly less radial dilution during the separation process compared to conventional LC; for instance a 0.1 mm ID column should give a theoretical 400-fold signal increase compared to a 2.1 mm ID column. Additional sensitivity can be achieved by large volume injection using an online solid phase extraction

**Abbreviations:** LC, liquid chromatography; ID, column inner diameter; nanoLC, nano scale liquid chromatography; microLC, micro scale liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; nanoESI, nano electrospray ionization; 25-GT, girard T derivative of 25-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 22S-OHC, 22S-hydroxycholesterol; MeOH, methanol; FA, formic acid; IPA, 2-propanol; AF, ammonium formate; AF/FA, 2.5 mM ammonium formate and 0.25% formic acid; SPE, solid phase extraction.

\* Corresponding author. Tel.: +47 22 85 55 87.

E-mail address: hanne.roberg-larsen@kjemi.uio.no (H. Roberg-Larsen).

<http://dx.doi.org/10.1016/j.steroids.2015.01.023>

0039-128X/© 2015 Elsevier Inc. All rights reserved.

Please cite this article in press as: Roberg-Larsen H et al. Underivatized oxysterols and nanoLC–ESI–MS: A mismatch. Steroids (2015), <http://dx.doi.org/10.1016/j.steroids.2015.01.023>

(SPE) column [11]. Nanospray ESI (nanoESI) allows for a larger percentage of the analytes to enter the MS, enhancing sensitivity [12,13], in addition to produces smaller charged droplets, which gives less ion suppression [14]. Although nanoLC–ESI–MS is more technically demanding to operate it is increasingly employed, particularly in proteomics, where it has become a standard approach. In metabolomics, the use of nanoLC is still very limited. However, nanoLC–ESI–MS can allow analysis of very small samples, and attomolar concentrations of 24S-OHC, 25-OHC and 27-OHC (derivatized with Girard T reagent) could be measured in just 10,000 pancreatic cancer cells, with excellent repeatability [8].

A disadvantage of charge-tagging oxysterols is that it can be time consuming (reactions can require elevated temperatures and overnight treatment), and may require considerable manual efforts, although several steps e.g. SPE clean-up (for removal of excess derivatization reagent) can be automated [8,15]. We therefore wanted to investigate whether the enhanced sensitivity of nanoLC–ESI–MS could compensate for the poor ionization of underivatized oxysterols, to allow sufficient detection of oxysterols in limited samples (e.g. million-scale cell numbers) in a shorter time than our present method [8]. This was partly inspired by the study of McDonald et al. [10] who could monitor oxysterol-salt complexes (along with a number of other lipids) in biological samples using ESI and an AB Sciex API 5000 triple quadrupole mass spectrometer without derivatization. Other ionization sources such as APPI [16] and APCI [17,18] were not considered as they are not compatible with the low flow used in nanoLC.

In the present study, the traits of nanoLC–ESI–MS for detecting Girard “charge-tagged” oxysterols and underivatized oxysterols have been compared, using two contemporary mass spectrometers (Q-Exactive™ hybrid quadrupole-Orbitrap and TSQ Quantiva™ triple quadrupole). MicroLC–ESI–MS methods for native and derivatized oxysterols using a column with similar reversed phase material were included for comparison.

## 2. Experimental

### 2.1. Chemicals and reagents

For microLC and nanoLC of derivatized oxysterols mobile phase A consisted of 0.1% Formic acid (FA) in type 1 H<sub>2</sub>O (Millipore, Billerica, MA, USA) and mobile phase B consisted of 0.1% FA in MeOH (Hipersolv grade, VWR, Radnor, PA, USA). For determination of native oxysterols, mobile phase A consisted of 2.5 mM ammonium formate (LC-MS quality, Fluka, Sigma Aldrich, St. Louis, MO, USA) and 0.25% FA (AF/FA) in type 1 H<sub>2</sub>O and mobile phase B consisted of AF/FA in MeOH, ethanol (EtOH, absolute, AnalaR Normapure, VWR) or 2-propanol (IPA, p.a., Sigma Aldrich). Ammonium acetate and acetic acid (both of LC–MS quality, Fluka, Sigma Aldrich) were also used as mobile phase additives.

A stock solution of 188 µg/mL cholest-5-ene-3β, 25-diol (25-OHC, Sigma Aldrich) was prepared by dissolving 25-OHC in 2-propanol (Rathburn chemicals Ltd., Walkerburn, Scotland, UK). This stock solution was diluted with mobile phase (50% B) to suitable concentrations before injections.

Other standard solutions for Girard T derivatives were prepared as previously described in [8].

### 2.2. Direct infusion

A solution of 1 µg/mL 25-OHC in IPA was derivatized to 25-GT with Girard T reagent as described elsewhere [8]. To remove excess Girard T reagent, a 100 mg C<sub>18</sub> Isolute (Biotage, Uppsala, Sweden) SPE column was used. The SPE column was conditioned with 1 mL MeOH followed by 1 mL type 1 H<sub>2</sub>O and 2 mL MeOH<sup>+</sup> type

1 H<sub>2</sub>O (1 + 1). The derivatized standard solution (700 µL in MeOH) was applied and the flow-through collected. The flow-through was diluted to approximately 30% MeOH and reappplied on the SPE column followed by a wash with 2 mL type 1 H<sub>2</sub>O. The 25-GT was eluted using 1 mL MeOH and 1 mL chloroform (AnalaR, VWR), evaporated into dryness and re-dissolved in 700 µL 0.1% FA in MeOH.

Solution for direct infusion of the native oxysterol 25-OHC was diluted from IPA stock solution with AF/FA in MeOH.

The solutions were infused with a flow rate of 1 µL/min using a direct infusion pump, 30 µm ID silanized fused silica tubing (see below), 30 µm ID stainless steel emitters and a nanospray flex ion source on both Q-Exactive™ hybrid quadrupole-Orbitrap and TSQ Quantiva™ triple quadrupole mass spectrometers (Both from Thermo Scientific, Waltham, MS, USA).

### 2.3. LC–MS

MicroLC of Girard T derivatives was performed with the same chromatographic condition as described in [15], but detection was performed with the Q-Exactive™ hybrid quadrupole-Orbitrap. The injected solution for microLC contained 14 nM 24S-GT, 25-GT, 27-GT and 22S-GT. For nanoLC the injected solutions contained 30 pM 24S-GT, 25-GT, 27-GT and 22S-GT and nanoLC of Girard T derivatives of oxysterols were performed with the same equipment and same chromatographic conditions as previously described [8].

For native oxysterols a 1 mm ID × 100 mm ACE 3 C<sub>18</sub>-PFP column was used in microLC, while a 0.1 mm ID × 100 mm ACE 3 C<sub>18</sub> column was used in nanoLC, as C<sub>18</sub>-PFP was not available in nano-dimensions at the time of study. The microLC flow rate was 40 µL/min and 1 µL of 800 ng/mL 25-OHC was injected. For nanoLC of native oxysterols, a column switching system as described in [8] was used. Loading mobile phase was mobile phase A (5 µL/min) and all tubing were silanized by flushing them with 5% chlorotrimethylsilane (Sigma Aldrich) in n-heptane (p.a., Merck, VWR) as described in [19]. The nanoLC flow rate was 500 nL/min and 2 µL of 15 ng/mL 25-OHC was injected. In micro LC the mobile phase composition went from 70% to 100% B in 15 min and was held at 100% B for 10 min, while in nanoLC the mobile phase composition went from 70% to 100% B in 20 min and was held at 100% B for 20 min.

### 2.4. Carry-over

To localization carry-over effects, a variety of solutions, columns and equipment were used. For details see Supplementary Table S1.

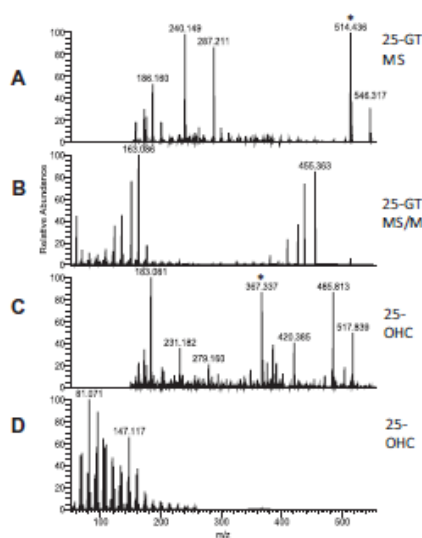
## 3. Results

The goal of our study was to develop a fast and easy nanoLC–MS method for determination of oxysterols in small cell samples. Preliminary experiments were conducted with different side-chain hydroxylated oxysterols (e.g. 24S-OHC, 27-OHC) and deuterated internal standard. However, as all these compounds behaved similar in the nanoLC–MS system, only results for 25-OHC is shown in the following section.

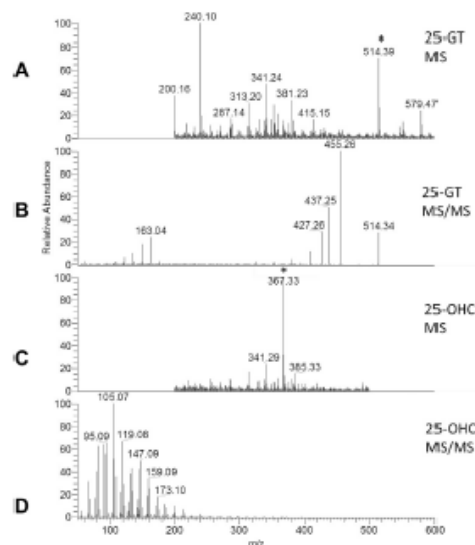
### 3.1. Adduct formation and fragmentation of native and derivatized oxysterols

Direct infusion of 25-GT in 0.1% FA in MeOH using a Q-Exactive™ hybrid quadrupole-Orbitrap with full MS mode (Fig. 1A) showed ample signal of the desired molecular ion *m/z* 514.436 [25-GT]<sup>+</sup>. MS/MS fragmentation with the HCD collision





**Fig. 1.** (A) MS spectrum ( $m/z$  200–550) of 1  $\mu\text{g/mL}$  25-GT in 0.1% FA in MeOH and (B) MS/MS spectrum of  $m/z$  514.4362 ( $[25\text{-GT}]^+$ ) on Q-Exactive™ hybrid quadrupole-Orbitrap (Resolution 140,000). (C) MS spectrum ( $m/z$  200–550) of 1  $\mu\text{g/mL}$  25-OHC in AF/FA in MeOH and (D) MS/MS spectrum of  $m/z$  367.3369 ( $[M+H-2H_2O]^+$ ) on Q-Exactive™ hybrid quadrupole-Orbitrap (Resolution 140,000).



**Fig. 2.** (A) MS spectrum ( $m/z$  200–550) of 1  $\mu\text{g/mL}$  25-GT in 0.1% FA in MeOH and (B) MS/MS spectrum of  $m/z$  514.4362 ( $[25\text{-GT}]^+$ ) on Quantiva™ triple quadrupole (Resolution 0.2 Da). (C) MS spectrum ( $m/z$  200–550) of 25-OHC and (D) MS/MS spectrum of  $m/z$  367.3369 ( $[M+H-2H_2O]^+$ ) on Quantiva™ triple quadrupole (Resolution 0.2 Da).

cell (Fig. 1B) produced a main fragment 455.363 (cleavage of Girard T [20]) suitable for quantification by SRM. Virtually the same mass spectrometry was observed using the Quantiva™ triple quadrupole (CID collision cell) (Fig. 2A/B).

Solvents/mixtures used for attempting to achieve stable ions of native oxysterols are shown in Table 1. Neither the Q-Exactive™ hybrid quadrupole-Orbitrap nor the TSQ Quantiva™ triple quadrupole (equipped with a nanoESI source) were able to produce adducts in positive mode as described by McDonald et al. [10]. Some adduct formation ( $[M+COO]^+$ ,  $m/z$  461.36) was observed using 10 mM ammonium acetate and negative ionization; however the signal intensity varied substantially from day to day. With 25-OHC solved in 0.1% FA in MeOH, loss of one or two water molecules ( $[M+H-H_2O]^+$  and  $[M+H-2H_2O]^+$ ,  $m/z$  385.347 and 367.337) was observed, but the signal intensity varied significantly. Direct infusion of 25-OHC in AF/FA in MeOH (Fig. 1C) showed stable and repeatable ions with loss of one or two water molecules, allowing for MS/MS experiments to commence. The HCD-induced fragmentation was however characterized by difficult-to-interpret spectra with few obvious choices for SRM transitions for quantitation, likely due to the lack of charge retaining centers of the underivatized oxysterol; MS/MS spectrum of  $[M+H-2H_2O]^+$  is shown in Fig. 1D. This behavior is similar to that found previously for many other steroids, using CID-induced fragmentation [21]. Indeed, comparable mass spectrometry was observed using the Quantiva™ triple quadrupole (Fig. 2C/D), however less noise and background ions were observed in MS-mode compared to the Q-Exactive™ hybrid quadrupole-Orbitrap.

### 3.2. Carry-over

When introducing the native oxysterols into our nanoLC-MS system, extreme and persistent carry-over was observed for all solvent/mixtures investigated (see Table 1). These effects were not

observed with native oxysterols in our microLC system or when introducing Girard T derivatives in our microLC or nanoLC systems, as shown in Fig. 3; if carry-over of native oxysterols was caused by the stationary phases, it should have been observed in the microLC system as well. When stripping down the LC system to consist of only a pump, a manual injector and a nanoESI-MS, all connected with fused silica tubing, the system had large oxysterol accumulation and most probably self-adsorption of oxysterols to the fused silica walls. This is illustrated in Fig. 4A, showing multiple injections of 25-OHC followed by injections of blank (FA/AF in IPA); the oxysterol signal decreases with each injection (i.e. less reaches the detector as the compound is increasingly adsorbed) and after 10 blanks the carry-over is virtually the same. To reduce these effects and remove unspecific binding to silanol groups on the fused silica, silanization (or “endcapping”) of the tubing was performed [19], converting the hydroxyl group on the surface of the fused silica to methyl groups. This was successful for the simple “stripped” LC system, as shown in Fig. 4B; injections of 25-OHC followed by injections of blank solutions show no evidence of carry-over from the silanized fused silica. Based on these observations, all the fused silica tubings in the nanoLC system were silanized. These efforts did not completely remove carry-over issues from the system. Even with silanized fused silica tubings, the carry-over was again large when the whole system was assembled, but not as persistent, and traces of oxysterols could be removed after approximately 60 min of washing with AF/FA in IPA. A variety of attempts to locate and reduce carry-over in the system are described in Supplementary material Table S1; however none of these was successful.

### 4. Discussion

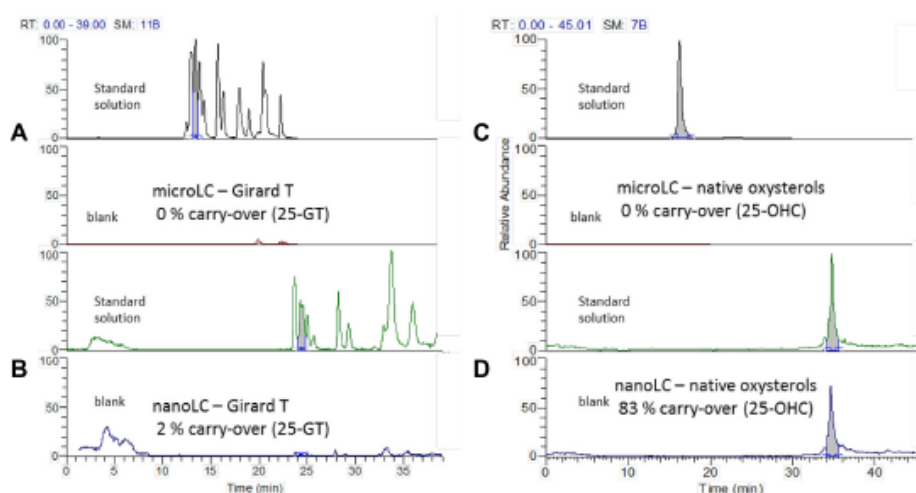
Mass spectrometry of underivatized oxysterol obtained with nanoESI was not convincing, as only unstable MS-and/or unclear



**Table 1**

Solvents used for MS and LC investigations.

Solvent direct infusion	Loading mobile phase	Eluting mobile phase	Results/comments
<b>MS investigations</b>			
10 mM ammonium acetate in MeOH, IPA or ACN	–	–	No stable adducts in positive MS mode
10 mM acetic acid in MeOH	–	–	No stable adducts from day to day in negative MS mode
0.1–0.25% FA in MeOH or IPA	–	–	No stable adducts in positive MS mode
2.5 mM AF in MeOH or IPA	–	–	No stable nanospray in negative mode
AF/FA in MeOH or IPA	–	–	Loss of one or two water molecules
10 mM AF in 70% ACN	–	–	Large variation in signal intensity
	–	–	Loss of one or two water molecules
	–	–	Large variation in signal intensity
	–	–	Loss of one or two water molecules
	–	–	Stable signal intensities
	–	–	No signal
<b>LC investigations</b>			
–	0.1% FA in H <sub>2</sub> O	0.1% FA in MeOH or IPA	Oxysterols trapped, large carry-over
–	0.1% FA in MeOH (<10%)	0.1% FA in MeOH or IPA	Oxysterols trapped, large carry-over
–	0.1% FA in MeOH (>10%)	0.1% FA in MeOH or IPA	No retention, carry-over not examined
–	0.1% FA in EtOH (>5%)	0.1% FA in EtOH	No retention, carry-over not examined
–	AF/FA in H <sub>2</sub> O	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over
–	AF/FA in MeOH (<10%)	AF/FA in MeOH or IPA	Oxysterols trapped, large carry-over
–	AF/FA in MeOH (>10%)	AF/FA in MeOH or IPA	No retention, carry-over not examined
–	–	0.1% FA in MeOH (<65%)	Solubility issues, poor peak shape
–	–	0.1% FA in MeOH (>65%)	Large carry-over
–	–	0.1% FA in IPA	Large carry-over
–	–	AF/FA in MeOH	Large carry-over
–	–	AF/FA in IPA	Large carry-over



**Fig. 3.** (A) Standard solution (14 nM 24S-GT, 25-GT, 27-GT and 22S-GT) injected on an ACE 3 C<sub>18</sub> 1 mm ID × 100 mm column using a column switching system as described in [15] followed by a blank injection. No carry-over was detected. (B) Standard solution (30 pM 24S-GT, 25-GT, 27-GT and 22S-GT) injected on an ACE 3 C<sub>18</sub> 0.1 mm ID × 100 mm column using a column switching system as described in [8], followed by a blank injection after injecting 30 cell samples without column cleaning (maximum 5 sample prior to cleaning is recommended to avoid carry-over [8]). Approximately 2% carry-over was found with these “worst case” conditions. (C) Standard solution (800 ng/mL) of 25-OHC injected on an ACE 3 C<sub>18</sub>-PFP 1 mm ID × 100 mm column. The PFP (pentafluorophenyl) groups in combination with C<sub>18</sub> are supposed to have shape selectivity hence more suitable for steroid isomers. No carry-over was detected. (D) Standard solution (15 ng/mL) 25-OHC injected on an ACE 3 C<sub>18</sub> 1 mm ID × 100 mm column using the column switching system as described in [15] followed by blank injection. All tubings were silanized. Gross carry-over was observed under these attempted “optimized” conditions. ACE 3 C<sub>18</sub>-PFP stationary phase for nano columns was not available.

MS/MS spectra were obtained. As a result, it would be difficult in our hands to employ this approach for reliable quantifications and/or scout for novel sterol compounds of biological relevance; one specific example is the ongoing search for an endogenous

oxysterol that controls the Hedgehog signaling pathway at the level of the Patched or Smoothened [4].

Regarding the gross carry-over of native oxysterols in nanoLC, extra-column material surfaces and connections are potent

Please cite this article in press as: Roberg-Larsen H et al. Underivatized oxysterols and nanoLC-ESI-MS: A mismatch. Steroids (2015), <http://dx.doi.org/10.1016/j.steroids.2015.01.023>

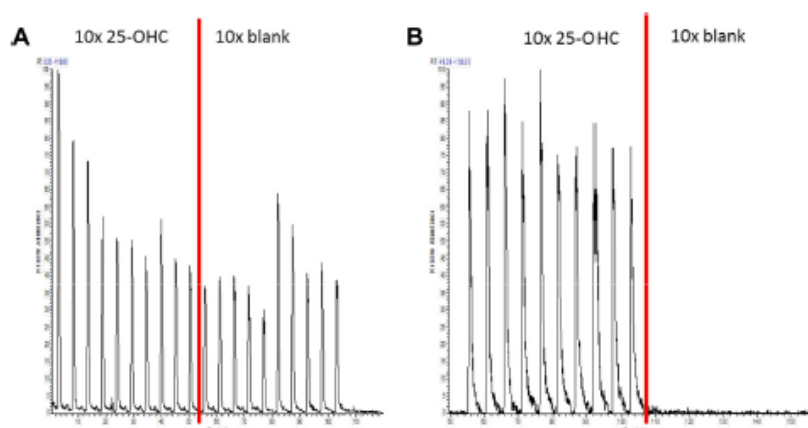


Fig. 4. Comparison of carry-over of oxysterols on (A) normal fused silica tubing and (B) silanized fused silica tubing.

contributors. This can be explained by e.g. the larger surface to volume ratio in the narrow fused silica tubings necessary in nanoLC to avoid band broadening compared to microLC tubing. Very narrow PEEK tubing was considered as an option, but in our experience, these tubings in nanoLC systems are associated with swelling/clogging issues. Other types of tubing suitable for nanoLC are to our knowledge not available, leaving fused silica tubing as the only option. An LC system with a larger column and tubing ID and similar stationary phase as in the nanoLC system did not suffer from carry-over effects. Thus, for measuring native oxysterols in larger samples (microbore/conventional columns e.g. [16,18,22]) should be safe to use regarding carry-over. On the other hand, for analysis of very limited samples (e.g. slow cycling cancer stem cells) our recommended choice would be to derivatize oxysterols with e.g. Girard reagents, prior nanoLC-ESI-MS.

Our observations also have implications for nanoLC-based studies that do not involve sterol determinations; the native oxysterols would adsorb to the surfaces of the materials of the system, with subsequent self-adsorption (even with the relatively low concentrations used). A consequence of this can be that oxysterols and possible other lipids can create extra-column pseudo-stationary phases, which can interfere with determination of other substances. Therefore, it is especially important in nanoLC systems to avoid unnecessary introduction of non-analyte sterols and these should be removed via e.g. off-line sample preparation. Similar problems have also been described for phospholipids [23], also regarding conventional LC systems. A possible step for reducing irreversible adsorption to the tubings, etc. of a miniaturized system is to pre-treat the fused silica capillaries employed, i.e. silanizing them. This process can significantly reduce carry-over, but nowhere near satisfactory; it seems that other surfaces of nanoLC systems also contribute to carry-over effects. We were however not able to locate these sites specifically. Using silanized capillaries, carry-over effects/adsorption of oxysterols could be removed washing with a solvent mixture of AF/FA in IPA, but more than 60 min were needed.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.steroids.2015.01.023>.

Please cite this article in press as: Roberg-Larsen H et al. Underivatized oxysterols and nanoLC-ESI-MS: A mismatch. *Steroids* (2015), <http://dx.doi.org/10.1016/j.steroids.2015.01.023>

#### References

- [1] Brown AJ, Jessup W. Oxysterols: sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis. *Mol Aspects Med* 2009;30:111–22.
- [2] Nelson ER, Wardell SE, McDonnell DP. The molecular mechanisms underlying the pharmacological actions of estrogens, SERMs and oxysterols: implications for the treatment and prevention of osteoporosis. *Bone* 2013;53:42–50.
- [3] Nelson ER, DuSelle CD, Wang X, Howe MK, Evans G, Michalek RD, et al. The oxysterol, 27-hydroxycholesterol, links cholesterol metabolism to bone homeostasis through its actions on the estrogen and liver X receptors. *Endocrinology* 2011;152:4691–705.
- [4] Roberg-Larsen H, Strand MF, Krauss S, Wilson SR. Metabolites in vertebrate hedgehog signaling. *Biochem Biophys Res Commun* 2014;446:669–74.
- [5] Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, et al. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. *Sci Trans Med* 2010;2:56ra81.
- [6] Karu K, Hornshaw M, Woffendin G, Bodin K, Hamberg M, Avelius G, et al. Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols. *J Lipid Res* 2007;48:976–87.
- [7] Honda A, Yamashita K, Hara T, Ikegami T, Miyazaki T, Shirai M, et al. Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. *J Lipid Res* 2009;50:350–7.
- [8] Roberg-Larsen H, Lund K, Vehus T, Solberg NT, Vesterdal C, Misaghian D, et al. Highly automated nanoLC-MS-based approach for thousand cell scale quantification of side chain-hydroxylated oxysterols. *J Lipid Res* 2014;55:1531–6.
- [9] Jiang X, Sidhu R, Porter FD, Yanjanin NM, Speak AO, de Vries DT, et al. A sensitive and specific LC-MS/MS method for rapid diagnosis of Niemann-Pick C1 disease from human plasma. *J Lipid Res* 2011;52:1435–45.
- [10] McDonald JG, Smith DD, Stiles AR, Russell DW. A comprehensive method for extraction and quantitative analysis of sterols and seosteroids from human plasma. *J Lipid Res* 2012;53:1399–409.
- [11] Roberg M, Malerod H, Roberg-Larsen H, Aass C, Wilson SR. On-line solid phase extraction-liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems. *J Pharm Biomed Anal* 2014;87:120–9.
- [12] Kebarle P, Verkerk UH. Electrospray: from ions in solution to ions in the gas phase, what we know now. *Mass Spectrom Rev* 2009;28:898–917.
- [13] Wilm M, Mann M. Analytical properties of the nanoelectrospray ion source. *Anal Chem* 1996;68:1–8.
- [14] Enke CG. A predictive model for matrix and analyte effects in electrospray ionization of singly-charged ionic analytes. *Anal Chem* 1997;69:4885–93.
- [15] Roberg-Larsen H, Strand MF, Grimmo A, Olsen PA, Dembinski JL, Rise F, et al. High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography-mass spectrometry. *J Chromatogr A* 2012;1255:291–7.
- [16] Ahonen L, Maire FBR, Savolainen M, Kopra J, Vreeken RJ, Hankemeier T, et al. Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure photoionization-mass spectrometry. *J Chromatogr A* 2014;1364:214–22.
- [17] Raith K, Brenner C, Farwanah H, Müller G, Eder K, Neubert RHH. A new LC/APCI-MS method for the determination of cholesterol oxidation products in food. *J Chromatogr A* 2005;1067:207–11.

- [18] Baila-Rueda L, Cenarro A, Cofan M, Orera I, Barcelo-Batlloiri S, Pocovi M, et al. Simultaneous determination of oxysterols, phytosterols and cholesterol precursors by high performance liquid chromatography tandem mass spectrometry in human serum. *Anal Methods* 2013;5:2249–57.
- [19] Seed B. Silanizing glassware. *Current protocols in immunology*. John Wiley & Sons, Inc.; 2001.
- [20] Griffiths WJ, Hornshaw M, Woffendin G, Baker SF, Lockhart A, Heidelberger S, et al. Discovering oxysterols in plasma: a window on the metabolome. *J Proteome Res* 2008;7:3602–12.
- [21] Guan F, Soma LR, Luo Y, Uboh CE, Peterman S. Collision-induced dissociation pathways of anabolic steroids by electrospray ionization tandem mass spectrometry. *J Am Soc Mass Spectrom* 2006;17:477–89.
- [22] DeBarber AE, Lütjohann D, Merckens L, Steiner RD. Liquid chromatography–tandem mass spectrometry determination of plasma 24S-hydroxycholesterol with chromatographic separation of 25-hydroxycholesterol. *Anal Biochem* 2008;381:151–3.
- [23] Neville D, Houghton R, Garrett S. Efficacy of plasma phospholipid removal during sample preparation and subsequent retention under typical UHPLC conditions. *Bioanalysis* 2012;4:795–807.

## 7.2 Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols [65]

Supplemental Material can be found at:  
<http://www.jlr.org/content/suppl/2014/05/02/jlr.D048801.DC1.html>

methods

### Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols<sup>[5]</sup>

Hanne Roberg-Larsen,\* Kaja Lund,<sup>†,§</sup> Tore Vehus,\* Nina Solberg,<sup>†</sup> Caroline Vesterdal,\* Dorna Misaghian,\* Petter Angell Olsen,<sup>†</sup> Stefan Krauss,<sup>†</sup> Steven Ray Wilson,<sup>\*,§</sup> and Elsa Lundanes\*

Department of Chemistry,\* University of Oslo, Oslo, Norway; Unit for Cell Signaling, Cancer Stem Cell Innovation Centre (SFI-CAST),<sup>†</sup> Oslo University Hospital, Rikshospitalet, Oslo, Norway; and Affitech Research AS,<sup>§</sup> Oslo, Norway

**Abstract** Iso-octyl chain-hydroxylated oxysterols were determined in attomoles per 10,000 cells concentrations in 10,000–80,000 cultured pancreatic adenocarcinoma cells, using a sensitive, highly automated nano-LC-ESI-MS-based method. Identified oxysterols included 24S hydroxycholesterol (24S-OHC), 25 hydroxycholesterol (25-OHC), and 27 hydroxycholesterol (27-OHC), while 20S hydroxycholesterol and 22S hydroxycholesterol were not detected. Lower mass limit of quantification was 23 fg (65 amol) for 25-OHC and 27-OHC (100 times lower than our previous method) and 54 fg (135 amol) for 24S-OHC, after derivatization into Girard T hydrazones and online sample cleanup using simplified and robust automatic filtration and filter back flushing solid phase extraction LC/MS/MS. The instrument configuration was easily installed using a commercial nano-LC/MS system. Recoveries in spiked sample were 96, 97, and 77% for 24S-OHC, 25-OHC, and 27-OHC, with within- and between-day repeatabilities of 1–21% and 2–20% relative SD, respectively. The study demonstrates the potential of nano-LC in lipidomics/sterolomics.—Roberg-Larsen, H., K. Lund, T. Vehus, N. Solberg, C. Vesterdal, D. Misaghian, P. A. Olsen, S. Krauss, S. R. Wilson, and E. Lundanes. Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols. *J. Lipid Res.* 2014. 55: 1531–1536.

**Supplementary key words** 24S hydroxycholesterol • 25 hydroxycholesterol • 27 hydroxycholesterol • cholesterol 24-hydroxylase • cholesterol 25-hydroxylase • cholesterol 27-hydroxylase

Oxysterols, which are hydroxylated derivatives of cholesterol, have been implied as activators of the Hedgehog (Hh) signaling pathway through binding to the Smoothened (SMO) receptor (1, 2). The degree of oxysterol activation of SMO is isomer dependent, with iso-octyl chain hydroxylated oxysterols (Fig. 1A) clearly being the most active (3, 4). In addition, the –OH position on the octyl

chain is also of great importance for activity [e.g., 25 hydroxycholesterol (25-OHC) is a stronger agonist than 24S hydroxycholesterol (24S-OHC)] (3, 4). Methods are called for to quantify endogenous oxysterol isomers (which are often very low abundance) to fully understand their roles and regulation in Hh signaling (1, 5). In this context, it is advantageous that a method is compatible with small samples (e.g., aggressive tumor cell side populations that are associated with altered signal pathway activity) (6).

In recent years, LC/MS methods have gained popularity for measuring oxysterols. Due to their neutral nature, oxysterols are not easily ionized with ESI, the most common ionization source in LC/MS. The ionization issue has been solved by, for example, charge tagging of the oxysterols into, for example, picolinyl esters (7), Girard derivatives (8–10), and *N,N*-dimethylglycine esters (11), which enhances sensitivity and selectivity. However, most methods based on the above-mentioned approaches are not optimal for limited cell/tissue samples, as they typically include extensive manual preparation steps and/or analyte-diluting conventional bore LC.

We have developed a nano-LC-based method enabling quantification of Hh active oxysterols in small samples (10,000–80,000 cells). Features of this method include robust, automatic sample purification, one-vial sample preparation, MS-based monitoring for autooxidation, high chromatographic selectivity, targeted mass spectrometric

Abbreviations: ACN, acetonitrile; AFL, automatic filtration and filter back flushing; cLOD, concentration limit of detection; Hh, Hedgehog; ID, inner diameter; RP, reversed phase; RSD, relative SD; SPE, solid phase extraction; 20S-OHC, 20S hydroxycholesterol; 22S-OHC, 22S hydroxycholesterol; 24S-OHC, 24S hydroxycholesterol; 25-OHC, 25 hydroxycholesterol; 27-OHC, 27 hydroxycholesterol.

<sup>†</sup>To whom correspondence should be addressed.

e-mail: [sevenw@kjemi.uio.no](mailto:sevenw@kjemi.uio.no)

<sup>[5]</sup> The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of nine supplementary data sections and one animation.

Manuscript received 3 March 2014 and in revised form 23 April 2014.

Published, JLR Papers in Press, May 2, 2014

DOI 10.1194/jlr.D048801

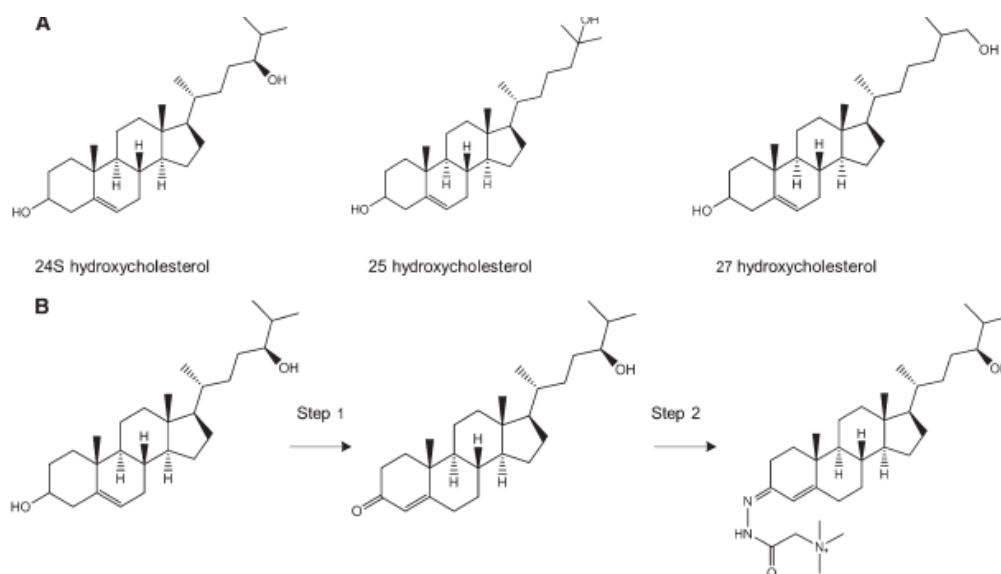
Copyright © 2014 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at <http://www.jlr.org>

Journal of Lipid Research Volume 55, 2014 1531

Downloaded from www.jlr.org at UNIVERSITETET I OSLO on February 26, 2015





**Fig. 1.** A: The structures of the Hh active oxysterols 24S-OHC, 25-OHC, and 27-OHC. B: Charge tagging of 24S-OHC with Girard T reagent. In step 1, the 3 $\beta$ -hydroxy-5-ene is enzymatically oxidized to 3-oxo-4-ene with cholesterol oxidase (1 h, 37°C). In step 2, the Girard T reagent reacts with the 3-oxo group to form Girard T derivatives of oxysterol. See Materials and Methods for more details.

detection, and a 100-fold increased mass sensitivity compared with our previous efforts (9). The targeting method enabled identification and quantification of Hh active 24S-OHC, 25-OHC, and 27 hydroxycholesterol (27-OHC) in cultured pancreatic adenocarcinoma cells (cell line BxPC-3). The compounds 20S hydroxycholesterol (20S-OHC) and 22S hydroxycholesterol (22S-OHC) were not found to be present above the detection limit in our samples.

## MATERIALS AND METHODS

### Chemicals and cell lines

All chemicals, reagents, and cells were from commercial suppliers and of analytical grade. See supplementary Section 1 for details of chemicals, suppliers, and cell culturing.

### Preparation of standard solutions, calibration solutions, validation samples, and cell samples

**Standard solutions and samples for method development and validation.** Standard solutions containing 13, 27, 54, 81, and 108 pM of each oxysterol, with 30 pmol cholesterol-25,26,27-<sup>13</sup>C as autooxidation-monitoring standard, were prepared from working solutions (1 nM).

For preparation of spiked validation samples, 10,000 cells were lysed in 300  $\mu$ l absolute ethanol (Kemetyl, Vestby, Norway) containing 120 pmol cholesterol-25,26,27-<sup>13</sup>C and spiked with standard solutions to give a concentration of 27, 54, and 108 pM of each oxysterol.

**Calibration solutions for quantification.** Calibration solutions were prepared from 1 nM working solutions and covered the concentration range 13–108 pM of each oxysterol. New calibration solutions were made for each assay.

**Cell samples.** Cells (10,000–80,000) were lysed in 300  $\mu$ l absolute ethanol (Kemetyl) containing 120 pmol cholesterol-25,26,27-<sup>13</sup>C. The samples were stored at –80°C before derivatization of oxysterols into Girard T derivatives.

**Internal standard.** Aliquots of 50  $\mu$ l of 1.5 nM internal standard working solution were added to all standard, calibration, and validation solutions and cell samples before evaporation into dryness and derivatization with Girard T as described subsequently.

### Charge tagging of oxysterol

Standard and calibration solutions, as well as cell and validation samples, were charge tagged with Girard T reagent as described previously (9) with small changes to adapt to the change in sample size; after evaporation into dryness, the residue was redissolved in 20  $\mu$ l 2-propanol. Aliquots of 200  $\mu$ l of 30  $\mu$ g/ml cholesterol oxidase dissolved in 50 mM phosphate buffer pH 7 were added to all solutions and samples to convert the 3 $\beta$ -hydroxy-5-ene to a 3-oxo-4-ene (Fig. 1B, step 1), performed at 37°C for 1 h using a Grant-Bio PHMT thermoshaker (Grant Instruments, Cambridge, UK) set to 300 rpm. To attach the Girard T reagent (Fig. 1B, step 2), 500  $\mu$ l of a mixture consisting of 15 mg Girard T reagent, 15  $\mu$ l glacial acetic acid, and 485  $\mu$ l methanol was added to the sample. This resulted in a final volume of 720  $\mu$ l of all solutions. The reaction was completed in the dark at room temperature overnight. Schematic view of the sample preparation is shown



Supplemental Material can be found at:  
<http://www.jlr.org/content/suppl/2014/05/02/jlr.D048801.DC1.html>

in supplementary Section II. Derivatized samples were stored at 4°C and analyzed within a week.

#### Automatic filtration and filter back flushing/solid phase extraction/nano-LC

Sample cleanup was performed online using automatic filtration and filter back flushing (AFFL) and online solid phase extraction (SPE), coupled upstream to nano-LC-ESI-Q Exactive MS. With the AFFL setup (Fig. 2 and supplementary Animation I) (12, 13), sample with excess reagent was injected (5  $\mu$ l) by an Agilent G1377A micro well autosampler (Agilent Technologies, Santa Clara, CA) (Fig. 2, step 1). An Agilent 1100 series pump was used as loading pump and filter back-flush pump [15  $\mu$ l/min of 0.1% formic acid (aq)]. Cell debris and precipitates/particulate matter from cells were trapped online on a 1  $\mu$ m Valco (Houston, TX) stainless steel filter (1/16", 1  $\mu$ m screen) fitted in a Valco union (1/16", 0.25 mm bore), while a reversed phase (RP) HotSep Tracy C<sub>8</sub> trap column [0.3 mm inner diameter (ID)  $\times$  5 mm] from G and T Septeck (Ytre Enebakk, Norway) trapped derivatized oxysterols (Fig. 2, step 2). Excess reagent, not sufficiently hydrophobic to be trapped by the RP trap column, was flushed to waste. The valve was subsequently switched after 3.5 min, and the loading pump automatically redirected to back flush the filter unit with 0.1% formic acid (aq) to clean the filter prior to the next injection (Fig. 2, step 3). Simultaneously, an Agilent 1200 series pump eluted the oxysterols from the trap column onto the ACE 3 C<sub>18</sub> (0.1 mm ID  $\times$  150 mm) RP analytical column (Advanced Chromatography Technologies, Aberdeen, UK) for isocratic separation using a mobile phase consisting of formic acid-water-methanol (0.1:5:95 v/v/v %) (Fig. 2, step 3) at a flow rate of 500 nL/min. Cholesterol residues in the column might accumulate and make a pseudostationary phase (14) and hence disturb the chromatographic separation. To remove cholesterol residues from column, the column was washed with formic acid-water-acetonitrile (ACN) (0.1:5:95 v/v/v %) (40 column volumes) after approximately six injections of cell samples to avoid changing the separation properties of the column. The column switching was performed with a 10-port two-position switching valve from Valco (1/16", 0.25 mm bore) controlled by the LC pump's Chemstation software.

#### MS of oxysterols

MS detection was performed in MS2 mode using a Q Exactive Orbitrap with nanospray flex ion source (Thermo Scientific,

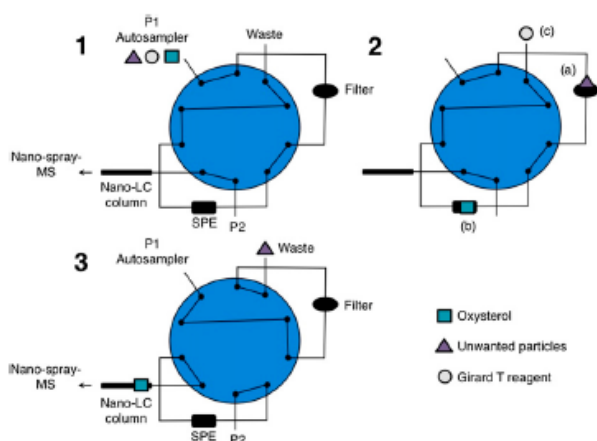
Waltham, MA). However, other mass spectrometers (e.g., triple quadrupole) would also be suitable. Ionization was performed in positive mode using a capillary voltage of 2.0 kV. The monitored MS/MS transitions were based on the fragmentation of the Girard T group (see supplementary Section III for illustration of the MS/MS fragmentation) and were 514.44  $\rightarrow$  455.36 (analytes), 520.40  $\rightarrow$  461.36 (internal standard), and 517.40  $\rightarrow$  458.36 (autooxidation monitoring). Collection of data and processing were performed using Xcalibur 2.2 software from Thermo Scientific. More MS instrument parameters (mostly based on recommended settings from the manufacturer) are found in supplementary Section IV.

## RESULTS AND DISCUSSION

Our goal was 2-fold: both enabling sensitive, simple, highly automated analysis of small samples (10,000 cell scale) and quantification of 27-carbon iso-octyl hydroxylated oxysterol analytes possibly present in cells. Candidates were 20S-, 22S-, 24S-, 25-, and 27-OHC, which are structural isomers with very similar LC/MS properties.

High sensitivity was achieved by using nano-LC and on-line sample preparation. Nano-LC columns (50–100  $\mu$ m ID) allow for compounds to elute in substantially smaller volumes compared with that in more conventional columns (e.g., 1 mm ID or larger). Therefore, analytes enter the ESI-MS in larger concentrations, resulting in enhanced signal. A nanobore column packed with the same stationary phase and particles as used in our previously published microbore LC-based method (9) was used, as the material has good oxysterol separation properties and durability. When replacing our previous microbore LC-ion trap MS methodology with nano-LC coupled with a Q Exactive MS, the mass limit of detection (and quantification) was reduced by a factor of 100 (from 2.5 pg to 23 fg). Separation properties (retention and selectivity) in the nano- and microbore format were similar, as expected.

The oxysterols were charge tagged with Girard T reagent (Fig. 1B, step 2). This established approach was chosen as



**Fig. 2.** AFFL using a 10-port two-position switching valve. Sample with excess reagent is injected by autosampler (1). A filter traps all cell debris and particulate matter (2), while Girard T derivates of oxysterols (more hydrophilic) are flushed into waste. After 3.5 min, the valve is switched automatically (3), and the filter is back flushed by the loading pump simultaneously as the Girard T derivatized oxysterols are eluted from the SPE and onto the nano-LC column for separation. See Materials and Methods for more details and supplementary Animation I. Monitoring of data started when the sample was injected.



the entire procedure can be performed in one tube, minimizing sample loss (9). In addition, it is compatible with automated SPE enrichment and cleanup (9).

The Girard T reagent reacts with the keto group as shown in Fig. 1B, step 2. A potential pitfall was that the LC/MS method would not distinguish between species with an already existing keto group and those with keto groups formed during sample preparation. However, analyte analogs with preexisting keto groups were not detected in the analyzed cell samples, investigated by performing Girard T tagging with and without step 1 in Fig. 1B (see supplementary Section V for chromatograms).

The 23 fg mass detection limit of this method corresponds to 13 pM in the vial (720  $\mu$ l). It is important to notice that the in-vial concentration will be affected by the available number of cells because the resulting total volume is the same for all samples and standard solutions after derivatization. Using more cells per sample will therefore give higher analyte amounts and concentrations, and hence a lower limit of detection per cell. For calculation example, see supplementary Section VI.

A 0.3 mm ID SPE column was coupled online with the nano-LC column. This avoids the overloading effects associated with direct injection of microliter amounts onto nano-LC columns (which can usually only handle nanoliter injections). Such overloading effects may call for extensive column purification routines; in the only previous nano-LC study of oxysterols (to the authors' knowledge), Karu et al. (10) had to inject a washing solvent between each sample injection to avoid carryover effects. In SPE-nano-LC mode, however, 5  $\mu$ l injections could easily be SPE trapped, and concentrated analyte bands were subsequently transferred to the LC column in appropriate volumes for satisfactory chromatographic resolution without carryover effects. In addition to allowing rather large injection volumes, online SPE greatly simplifies analysis of our 10,000 cell-scale samples, as excess Girard reagent removal is performed online. This reduces difficult, manual handling of minute samples/fractions, which can be a source of analysis variance (15).

Conventional online SPE-nano-LC can be especially prone to pressure buildups due to the small, relatively easily clogged connections. To avoid this, an AFFL setup was installed upstream to the SPE step. This allows crude samples to be injected directly (here: unfiltered lysates) as remaining cell debris and so forth is trapped on a stainless steel filter and washed off of the system after each sample injection. This reduces off-line sample preparation steps (and hence possible loss of analyte). The AFFL-SPE-nano-LC/MS methodology could handle thousand injections of relatively unprepared samples, representing an unprecedented robustness regarding nano-LC-based instrumentation. Our AFFL-SPE-nano-LC system can be assembled using a commercial SPE-LC system [see (16) for various systems], only requiring replacement of one valve (10 port instead of a 6 port) (13), compared with our original system, which required an additional pump (12).

An additional simplifying aspect of the method was the absence of a (off-line) cholesterol removal step, commonly

performed to avoid autooxidation effects that can negatively affect method accuracy. Enzymatically formed side chain-hydroxylated oxysterols (our analytes) are not typically associated with autooxidation; on the other hand, little is known, for example, about the formation of 20S-OHC, as no known enzyme is associated with the formation of this compound. Nonetheless, heavy cholesterol (cholesterol-25,26,27- $^{13}$ C) was added to all samples and calibration solutions. If autooxidation occurred during sample preparation (turning cholesterol into our analytes), heavy cholesterol would be converted to heavy oxysterols. This mass transition was monitored and controlled for each sample with MS. Autooxidation(-like) effects were in fact observed for  $\sim$ 1 in every 500 samples (these samples were rejected).

The chromatographic resolution of our method revealed that, presumably due to generation of *syn/anti* forms during the derivatization process (7, 17), several of the analytes appeared as two peaks. As these peaks were well resolved, their areas could be combined for quantification. By using methanol as the LC organic modifier instead of ACN, we were able to quantitatively distinguish 24S-OHC and 27-OHC, which coeluted with ACN as modifier. In spiked samples, 27-OHC and 20S-OHC coeluted with methanol as the organic modifier, but it was possible to resolve 20S-OHC from the other oxysterols when using ACN (see supplementary Section VII for chromatograms). In accordance with other recent studies (9, 18, 19), 20S-OHC was not observed in the analyzed cell samples using ACN as mobile phase, even when the sample amount was increased 8-fold. Hence, methanol could be used as the organic modifier for our method for reliable quantification of 27-OHC.

In a pancreatic cell line (BxPC-3) and a derived clonal mutant cell line ( $\Delta\beta$ cat BxPC-3), the sensitivity of the method allowed detection of 24S-OHC, 25-OHC, and 27-OHC in as little as 10,000 cultured cells (Fig. 3). In contrast, 20S-OHC and 22S-OHC were not detected in the cells even when using larger samples. The compounds 24S-OHC, 25-OHC, and 27-OHC were quantified (38, 19, and 35 fmol/10,000 cells, respectively). In support of these oxysterols being generated in the cells was the within-cell presence of corresponding enzymes responsible for formation of these oxysterols, both at the transcriptional level by RT-PCR and the protein level by Western blot and nano-LC/MS/MS. Also, a consistent and significant upregulation of 27-OHC and its corresponding enzyme (cholesterol 27-oxidase) as function of a cell perturbation (i.e., knock-out of  $\beta$  catenin) was observed (for more details, see supplementary Section IX).

#### Validation of the nano-AFFL-SPE-LC/MS/MS method

The concentration limit of detection (cLOD) was defined as the lowest concentration that repeatably produced a chromatographic peak with signal-to-noise ratio  $>3$ . The concentration limit of quantification (cLOQ) was defined as the concentration that produced peak areas with a relative SD (RSD)  $\sim$ 20%. The cLODs were 13 pM derivatized analyte for 24S-OHC, 25-OHC, and 27-OHC, corresponding



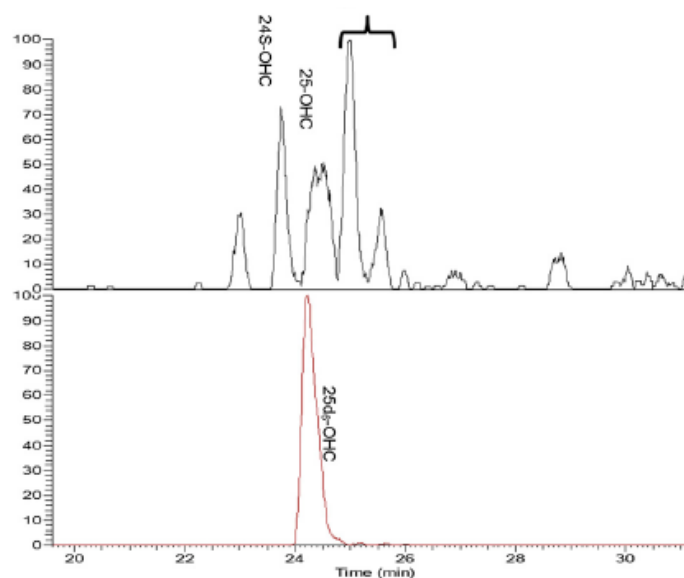


Fig. 3. Extracted ion chromatogram (EIC) from MS/MS analysis of derivatized oxysterols ( $m/z$  514.44–455.36, top) and internal standard (25d<sub>5</sub>,  $m/z$  520.40–461.40, bottom) in 10,000 BxPC-3 cells.

to 23 fg (65 amol) in 5  $\mu$ l injected on column. cLOQ was also 13 pM for 25-OHC and 27-OHC, while for 24S-OHC, the cLOD was 27 pM (54 fg, 135 amol injected on column).

Linearity of the method in spiked samples was examined in the range 27–108 pM, and the correlation coefficient ( $r^2$ ) for all linearity curves was >0.99 for all analytes (three concentration points, three spiked samples per concentration point). The slope of the curves corresponded to that in standard solutions (five concentration points, three standard solutions per point). Standard solutions could therefore be used as calibration solutions. Ideally, calibration solutions should be based on cell samples, but this was not possible due to lack of cell matrix without oxysterols. The linear concentration range examined covered the concentration range observed in studied samples.

Repeatability was examined for low (13 pM), medium (54 pM), and high (108 pM) concentration levels. Three individually prepared standard solutions per level were injected over 4 days (total of 12 standard solutions for each concentration level) for all cell-detected analytes. Within-day repeatability at low concentration level was between 2% and 21% RSD for 25-OHC and 27-OHC. The between-day repeatability was between 15% and 20% RSD for 25-OHC and 27-OHC (but somewhat higher for 24S-OHC, 27% RSD) at low concentration level and 2–6% RSD at medium and high levels for all analytes (4 days).

Recovery [or more correctly, the apparent recovery (20)] was examined by comparing samples spiked at three concentration levels with standard solutions of the same concentration levels, low (27 pM), medium (54 pM), and high (108 pM). The recovery was 96, 97, and 77% for 24S-OHC, 25-OHC, and 27-OHC, respectively. See supplementary Section VIII for more details (linearity curves, equations, etc.).

## CONCLUSIONS

A highly sensitive, robust, and automated method for quantification of the natural iso-octyl chain hydroxylated oxysterols 24S-OHC, 25-OHC, and 27-OHC was successfully developed and validated and applied to measure oxysterols in small samples ( $\geq 10,000$  cells). The study illustrates robust and successful use of nanoscale LC in targeted lipidomics/sterolomics, an approach that is usually “reserved” for proteomics. The identification/presence of the endogenous oxysterols in BxPC-3 cells was supported by identification of corresponding enzymes responsible for formation of these oxysterols within the cells. The method will be further used in studying endogenous side chain-hydroxylated oxysterols in the Hh pathway in the context of cancer.



Supplemental Material can be found at:  
<http://www.jlr.org/content/suppl/2014/05/02/jlr.D048801.DC1.html>

## REFERENCES

- Nachtergaele, S., L. K. Mydock, K. Krishnan, J. Rammohan, P. H. Schlesinger, D. F. Covey, and R. Rohaig. 2012. Oxysterols are allosteric activators of the oncoprotein Smoothened. *Nat. Chem. Biol.* **8**: 211–220.
- Roberg-Larsen, H., M. F. Strand, S. Krauss, and S. R. Wilson. 2014. Metabolites in vertebrate Hedgehog signaling. *Biochem. Biophys. Res. Commun.* In press.
- Corcoran, R. B., and M. P. Scott. 2006. Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc. Natl. Acad. Sci. USA.* **103**: 8408–8413.
- Dwyer, J. R., N. Sever, M. Carlson, S. F. Nelson, P. A. Beachy, and F. Parhami. 2007. Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. *J. Biol. Chem.* **282**: 8959–8968.
- Sharpe, H. J., and F. J. de Sauvage. 2012. Signaling: an oxysterol ligand for Smoothened. *Nat. Chem. Biol.* **8**: 139–140.
- Dembinski, J. L., and S. Krauss. 2009. Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreatic adenocarcinoma. *Clin. Exp. Metastasis.* **26**: 611–623.
- Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. 2009. Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. *J. Lipid Res.* **50**: 350–357.
- Griffiths, W. J., M. Hornshaw, G. Woffendin, S. F. Baker, A. Lockhart, S. Heidelberg, M. Gustafsson, J. Sjoval, and Y. Wang. 2008. Discovering oxysterols in plasma: a window on the metabolome. *J. Proteome Res.* **7**: 3602–3612.
- Roberg-Larsen, H., M. F. Strand, A. Grimsmo, P. A. Olsen, J. L. Dembinski, F. Rise, E. Lundanes, T. Greibrokk, S. Krauss, and S. R. Wilson. 2012. High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography-mass spectrometry. *J. Chromatogr. A.* **1255**: 291–297.
- Karu, K., J. Turton, Y. Wang, and W. J. Griffiths. 2011. Nano-liquid chromatography-tandem mass spectrometry analysis of oxysterols in brain: monitoring of cholesterol autooxidation. *Chem. Phys. Lipids.* **164**: 411–424.
- Jiang, X., D. S. Ory, and X. Han. 2007. Characterization of oxysterols by electrospray ionization tandem mass spectrometry after one-step derivatization with dimethylglycine. *Rapid Commun. Mass Spectrom.* **21**: 141–152.
- Svendsen, K. O., H. R. Larsen, S. A. Pedersen, I. Brenna, E. Lundanes, and S. R. Wilson. 2011. Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography. *J. Sep. Sci.* **34**: 3020–3022.
- Røen, B. T., S. R. Sellevåg, K. E. Dybendal, and E. Lundanes. 2014. Trace determination of primary nerve agent degradation products in aqueous soil extracts by on-line solid phase extraction - liquid chromatography - mass spectrometry using ZrO<sub>2</sub> for enrichment. *J. Chromatogr. A.* **1329**: 90–97.
- Ogden, P. B., and J. W. Coym. 2011. Retention mechanism of a cholesterol-coated C18 stationary phase: van't Hoff and Linear Solvation Energy Relationships (LSER) approaches. *J. Chromatogr. A.* **1218**: 2936–2943.
- Hyötyläinen, T. 2009. Critical evaluation of sample pretreatment techniques. *Anal. Bioanal. Chem.* **394**: 743–758.
- Roberg, M., H. Malerød, H. Roberg-Larsen, C. Aass, and S. R. Wilson. 2014. On-line solid phase extraction-liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems. *J. Pharm. Biomed. Anal.* **87**: 120–129.
- Griffiths, W. J., P. J. Crick, and Y. Wang. 2013. Methods for oxysterol analysis: past, present and future. *Biochem. Pharmacol.* **86**: 3–14.
- McDonald, J. G., D. D. Smith, A. R. Stiles, and D. W. Russell. 2012. A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma. *J. Lipid Res.* **53**: 1399–1409.
- Myers, B. R., N. Sever, Y. C. Chong, J. Kim, J. D. Belani, S. Rychnovsky, J. F. Bazan, and P. A. Beachy. 2013. Hedgehog pathway modulation by multiple lipid binding sites on the Smoothened effector of signal response. *Dev. Cell.* **26**: 346–357.
- Burns, D. T., K. Danzer, and A. Townsend. 2002. Use of the term "recovery" and "apparent recovery" in analytical procedures (IUPAC Recommendations 2002). *Pure Appl. Chem.* **74**: 2201–2205.